Small molecule analysis on a modified hydrophilic interaction liquid chromatography (HILIC) monolith using

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Small molecule analysis on a modified hydrophilic interaction liquid chromatography (HILIC) monolith using micro-HPLC and capillary electrochromatography (CEC)

by

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Abstract

Monolithic columns fulfil the need for new chromatographic approaches to small-molecule analysis. These columns can be manufactured for nano-scale use, and are resistant to extreme pH conditions; however, they are less efficient than conventional silica-based materials. Efficient monoliths should conceivably be able to separate and analyse small molecules with better performance than conventional columns. The research investigated a development of new monolithic stationary phases for small molecule analysis using micro-high performance liquid chromatography (micro-HPLC) and capillary electrochromatography (CEC) techniques. The work was divided into three phases, as follow.

In the first phase, the validity of instruments and electroosmotic flow (EOF) proving were investigated. A linear relationship between applied flow rates and actual flow rates on DiNa Nano pump was detected. Evaluation involves a comparison of their theoretical plate numbers *(N)* and the performance with a group of suitable test compounds with a range of polarities, including highly polar compounds. HILIC behaviour study is also part of the phase. In this phase, however CE theoretically demonstrated acids and bases separations.

In the second phase, experimental and method optimisation were discovered. The optimised protocol of HILIC successfully worked with micro-HPLC but was not applicable with CEC. An EOF of materials for CEC was modified to resolve this drawback and described in this phase (Chapter 3, 4 and 5).

The key finding is that the modified HILIC monoliths, poly N, N-dimethyl-N-(2-methacryloyloxyethyl)-N-(3-sulphopropyl) ammonium betaine – co - ethylene glycol dimethacrylate – co - 2-acrylamido-2-methyl-1-propanesulfonic acid (SPE-co-EDMA-co-AMPS) generated the best performance on CEC. This best column was considered to apply on small molecule analysis for the third phase.

In the third phase, the utility of modified HILIC (SPE-co-EDMA-co-2%AMPS) monolithic columns for small-molecule analysis was demonstrated. Commercial chlorpheniramine maleate tablets were analysed using the modified monoliths and both micro-HPLC and CEC methods. The modified HILIC monolith showed excellent performance on both methods, with chlorpheniramine maleate measured in high reproducibility, specificity, pH stability, and accuracy, but performed better when used with CEC rather than micro-HPLC.

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List of symbols and abbreviations

A	Contribution of Eddy diffusion to band broadening
ACN	Acetonitrile
AIBN	2, 2-azobisisobutyronitrile
AMPS	2-acrylamido-2-methyl-1-propanesulfonic acid
A_s	Asymmetry factor or peak symmetry
B	Contribution of longitudinal diffusion to band broadening
BADMA	Bisphenol A dimethacrylate
BAEDA	Bisphenol A ethoxylate diacrylate
BGE	Background electrolyte
C	Contribution of resistance to mass transfer
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
cm	Centimetre
CPM	Chlorpheniramine maleate
CZE	Capillary zone electrophoresis
DMPA	2, 2-dimethoxy-2-phenylacetophenone
DMSO	Dimethyl sulfoxide
DVB/STY	Divinylbenzene-styrene
EDMA	Ethylene glycol dimethacrylate
EDMA/MMA	Ethylene dimethacrylate-methyl methacrylate

EOF	Electroosmotic flow
F	Volumetric flow rate
GC	Gas chromatography
GMA	Glycidyl methacrylate
GMA-EDMA	Glycidyl methacrylate-co-ethylenedimethacrylate
HEMA	2-hydroxyethyl methacrylate
H, *HETP*	The plate height, height equivalent to a theoretical plate
HILIC	Hydrophillic interaction chromatography
HILIC/SCX	Hydrophillic interaction chromatography with strong cationic exchange
HIPE	High Internal Phase Emulsion Polymerisation
H_{min}	Minimum of the plate height
HPLC	High performance liquid chromatography
i.d.	Internal diameter
k'	Capacity factor, or retention factor
kV	Kilovolt
L	Column length
L_d	Effective length
L_t	Total length
m	Meter
M	Molar (Molarity)

mBar	Millibar
MEKC	Micellar electrokinetic chromatography
MeOH	Methanol
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimetre
mmol	Millimole
mM	Millimolar
MPa	Megapascal
MS	Mass spectrometer
m/z	Mass to charge ratio
n	Number of sample
N	Normality (Normal)
N	Number of theoretical plate
NaOH	Sodium hydroxide
nL	Nanolitre
nm	Nanometre
N/m	Plate per metre
NP	Normal phase
o.d.	External diameter

Pa	Pascal
PDAM or PEDAS	Pentaerythritol diacrylate monostearate
PEDAS-co-AMPS	Poly (pentaerythritol diacrylate monostearate-co-2-acrylamido-2-methyl-1-propanesulfonic acid)
PEG	Poly (ethylene glycol)
PEO-PPO-PEO) or PPOPEO-PPO	Poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide)
pg	Pico gram
poly-GDMA	Poly (glycerol dimethacrylate)
PPG-PEG-PPG	Poly (ethylene glycol methyl ether acrylate-co-polyethylene glycol diacrylate)
PS	Polystyrene
PS/DVB	Polystyrene divinylbenzene
psi	Pound per square inch
pH	Potential of hydrogen
pKa	Potential of the negative decadic logarithm of the ionization constant of an acid
r	Radius of the column
RP	Reversed-phase
RPLC	Reverse phase liquid chromatography
R_s	Resolution
R^2	Correlation

SCX	Strong cationic exchange
SD	Standard deviation
SEM	Scanning Electron Microscopy
SFC	Supercritical fluid chromatography
SPE	N, N-dimethyl-N-(2-methacryloyloxyethyl)-N-(3-sulfopropyl) ammonium betaine
SPE-co-EDMA	Poly (N, N-dimethyl-N-(2-methacryloyloxyethyl)-N-(3-sulfopropyl) ammonium betaine-co- ethylene glycol dimethacrylate)
SPE-co-EDMA-co-AMPS	Poly (N, N-dimethyl-N-(2-methacryloyloxyethyl)-N-(3-sulfopropyl) ammonium betaine-co- ethylene glycol dimethacrylate-co-2-acrylamido-2-methyl-1-propanesulfonic acid)
THF	Tetrahydrofuran
t_o	Void time
t_m	Migration time
t_R	Retention time
TRIM	Trimethylolpropane trimethacrylate
TRIS	2-Amino-2-hydroxymethyl-propane-1,3-diol
u	Linear velocity
u_{opt}	Optimum linear velocity
UV	Ultraviolet
V	Applied voltage

V-501	4, 4’-azobis (4-cyanopentanoic acid)
w	Peak width at baseline
W	Watt
w_h	Peak height at half height
x	Contribution of longitudinal (X) axis of graph
y	Contribution of horizontal (Y) axis of graph
α	Selectivity
^{o}C	Degree celcius
γ-MAPS	3-(trimethyoxysilyl) propyl methacrylate
μ_{ep}	Mobility
μ_{app}	Measured mobility
μ_{eff}	Effective mobility
μ_{EOF}	Elecroosmotic flow mobility
µg	Microgram
µL	Microlitre
µm	Micrometre
π	Pi, mathematical constant, the ratio of a circle's circumference to its diameter, approximated as 3.14159
8-ODDMA	8-octanediol dimethacrylate
%RSD	Percent of relative standard deviation
%v/v	Percent volume by volume

%w/v	Percent weight by volume
%w/w	Percent weight by weight

Chapter 1

Introduction

Chapter 1. Introduction

In this chapter, fundamental chromatographic principles and techniques, including HPLC, micro-HPLC, CE and CEC, are reviewed. This is followed by a brief description of monolith properties, their preparation, and common applications. Small molecule analysis manuscripts using monoliths and nano-separation revision also included in the chapter. The aim of the research is also established. Poly organic hydrophilic interaction liquid chromatography (HILIC) monoliths were chosen as a core material for this research.

1.1 Background of Chromatography

Chromatography is a highly effective and widely used separation technique for either qualitative or quantitative analysis which has been researched for more than 100 years [1]. In 1903 the first chromatographic technique was introduced by Tswett reporting the separation of plant pigments including carotenoids, chlorophyll A and chlorophyll B by using normal phase chromatography [2].

Much research has been conducted into developing the separation technique in many aspects over the last 70 years. In 1941 liquid-liquid chromatography was developed by Martin and Synge the paper chromatography was carried out thereafter by Martin and colleagues [2]. There were many studies referring to and extensively analysing a wide range of compounds using high pressure liquid chromatography (HPLC) and in the 1950's the first commercial HPLC equipment was launched. Moreover, gas chromatography (GC) which uses gas as a carrier in

order to analyse volatile compounds was invented and widely used. In addition to these chromatographic techniques supercritical fluid chromatography (SFC) was introduced in 1958 and is still being developed [3].

Comparison with another very competitive technique, capillary electrophoresis (CE) was firstly reported in Tiselius' thesis in 1930 and the first commercial CE instrument appeared in the 1960's [2]. Thereafter the hybridisation between HPLC and CE was introduced as capillary electrochromatography (CEC) to cope with their advantages. Since the application of electroosmotic flow (EOF) on chromatographic column was introduced by Pretorius, there has been much research focusing on HPLC and CEC [4].

A high performance liquid chromatography (HPLC) column used in reversed-phase chromatography can present problems with peak shape and retention due to silanol interactions for some analytes, and since many drugs are basic, this is an issue in pharmaceutical analysis. Monolithic columns are a new type of separation media developed over the past decade that overcome these problems, and are increasingly replacing conventional packed columns in the analysis of basic compounds. They have a rigid sponge-like structure, permeated by pores which allow mass transfer of the mobile phase, reducing flow resistance compared to conventional packed columns, whilst maintaining good efficiency.

Over the past decade, a new group of separation media have been introduced in the form of the monolithic column. These are a type of stationary phase, different in structure to the traditional HPLC columns. In the latter, the column is packed

with inert particles, usually silica. The monolithic stationary phase, however, is a continuous solid backbone that is permeated by pores with a morphology analogous to that of a sponge [5]. The pores are of two types, macropores (~20 µm) and mesopores (~0.013 µm) [6]. This highly porous structure provides less flow resistance, thus allowing mass transfer of the mobile phase giving faster analysis times when compared to conventional silica-packed columns, as well as greater sensitivity and efficiency [7].

Furthermore, monolithic columns are structurally stable over a wider pH range than conventional silica based phases. Basic drugs are normally only neutral at high pH levels, above pKa values of the drugs. This stability at extreme pH ranges allows a more accurate analysis of basic drugs above their pKa_s in their neutral state, improving peak shapes and thus resolution. On the other hand in a conventional packed column, the silica particles are prone to degradation at more extreme pH levels, therefore it is more challenging to analyse basic drugs (small molecules) at these pH ranges because of the problems presented with peak tailing due to silanol interactions [8].The extra structural stability of monolithic columns also allows them to be sterilised using an autoclave [5].

Monolithic columns can be prepared relatively easily [9] in situ, further reducing costs, in contrast with the preparation of packed columns which is a highly skilled task. Monolithic columns can exist in various types: neutral, cationic, anionic or zwitterionic. In cation exchange, the analytes are positively charged and the column surface is negatively charged in order to provide electrostatic interaction between the two. Furthermore, the monolithic surface can be modified with a

variety of chemical moieties, and with the large chemistry bank available, it is possible to tailor the chemical interactions between the analytes and the column in order to optimise separation [7].

1.2 Micro separation techniques

1.2.1 Micro High Performance Liquid Chromatography (micro-HPLC)

Classical high performance liquid chromatography or high pressure liquid chromatography (HPLC) has been widely used by pharmaceutical companies since their invention. The improvement in sensitivity of detection, efficiency of separation, speed of analysis and cost-effectiveness of analysis were enhanced by the development of micro high performance liquid chromatography (micro-HPLC). There are basically four important components in a conventional HPLC system: a high pressure pump, a sample injector, a column and a detector, as shown in the following Figure 1.1 [10].

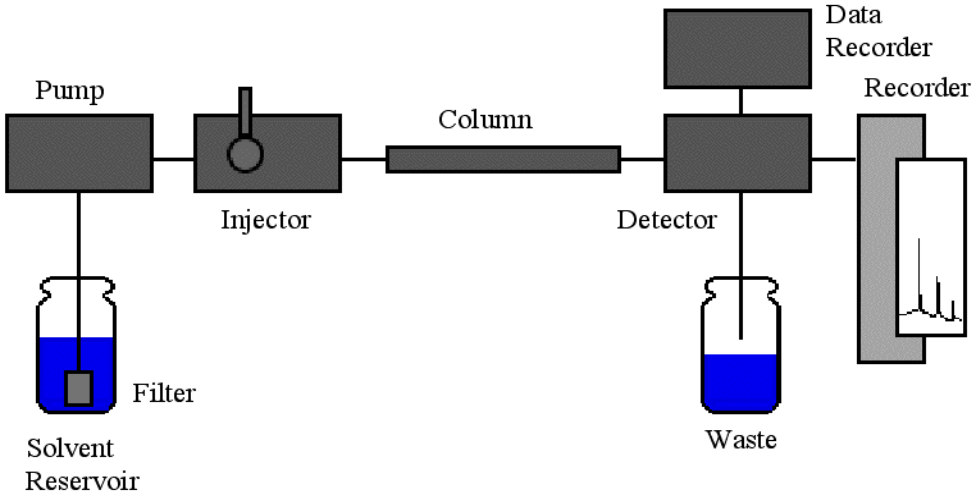


Figure 1.1 A typical HPLC instrument

Advanced micro-HPLC pumps can now deliver a very accurate low flow rate of liquid mobile phase at a micro to nano scale which is essential when used to pump through a capillary column in a micro system. In addition, reduction in the conventional column diameter, length and particle diameter (4.6 mm (i.d.) x 100 mm - 250 mm length packed with 3 to 5 µm particles) to a micro-HPLC column (0.8 to 2.1 mm (i.d.) x 50 mm length packed with <2 µm) should lead to a better performance. For example, there is a big sensitivity improvement using narrow inner diameter (i.d.) columns and these can more easily be coupled to other detectors including a mass-spectrometer (MS) [11].

1.2.2 Capillary Electrophoresis (CE) and Capillary Electrochromatography (CEC)

Capillary electrophoresis (CE) and capillary electrochromatography (CEC) are both micro separation techniques using the principle of electrophoresis for which an electroosmotic flow (EOF) is the essential driving force. Associated analytes can be separated in the capillary by the application of a voltage across its length. The mechanism involves the production of an electroosmotic flow resulting from a charge induced on the inner capillary wall in the case of capillary zone electrophoresis (CZE) or the surface of the stationary phase in CEC in the presence of a background electrolyte (BGE). In CZE, neutral and ionisable analytes (positive and negative ions) move through a capillary under an electric field and the separation mechanism is dictated by the different mass to charge ratio (m/z) of the analytes. However, because neutral analytes have no charge, it is not possible to resolve them using CZE [12]. However, micellar electrokinetic chromatography (MEKC) is a CE technique used for neutral compounds

separation. Neutral molecules as well as ionic species can be separated as a result of their solubilisation within the micelles, which form a pseudo-stationary phase analogous to the stationary phase in HPLC [13]. The following Figure 1.2 shows a diagram of a typical capillary zone electrophoresis (CZE) layout [14].

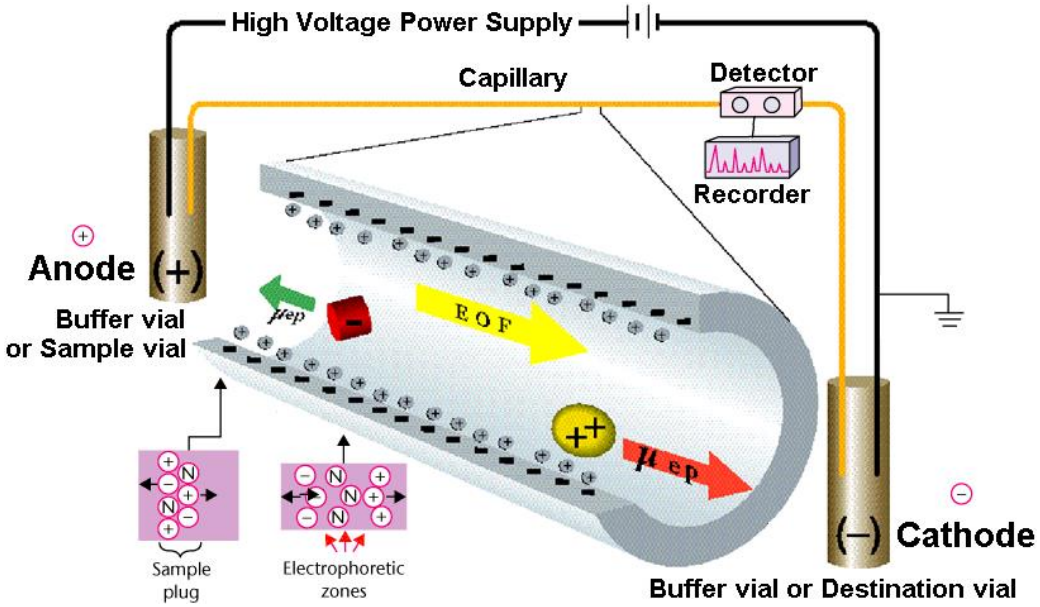


Figure 1.2 A capillary zone electrophoresis (CZE) layout (modified from Christopher Master's thesis 2008, [14])

Capillary electrochromatography (CEC) uses a capillary column containing a stationary phase which has capability of generating an electroosmotic driving force in a similar manner to that provided by the inner wall of the capillary itself. In addition, CEC is a hybrid separation method that couples the high separation efficiency of CZE with HPLC. An electric field is used in CEC to propel the mobile phase through a packed bed of a column rather than hydraulic pressure as used in HPLC. Consequently, this allows CEC to achieve very high efficiency. Because in CEC there is no backpressure, it is possible to use small diameter packings and

long packed beds. An additional benefit of CEC compared to HPLC is the fact that whilst the flow profile in a pressure driven system is parabolic, in an electrically driven system it is plug-like and therefore much more efficient (Figure 1.3). This is the source of highly efficient separations in CEC. There are many types of CEC including open tubular, packed capillary and some replaceable media filled capillaries [13].

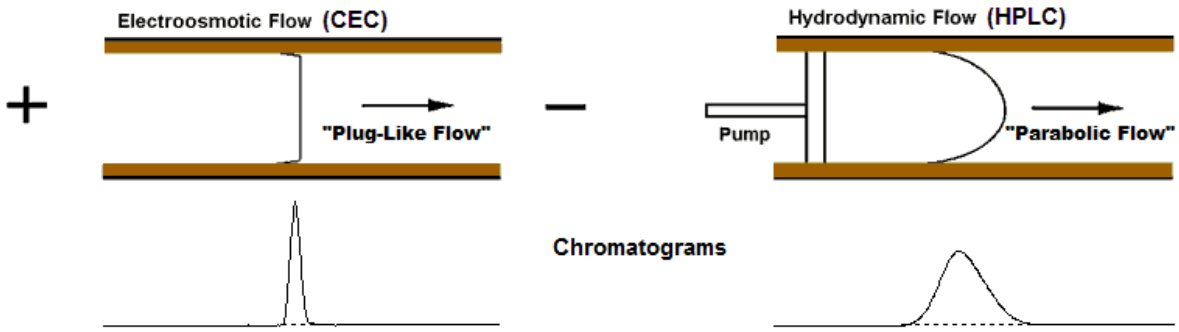


Figure 1.3 A plug-like flow (CEC) and a parabolic flow (HPLC)

Analytes will migrate as a result of many factors, for example, the applied voltage, the pH of the system, type of buffer, and buffer concentration. The EOF can be calculated from Equation 1 using thiourea or DMSO as an EOF marker.

$$\mu_{ep} = \frac{Ld / tm}{V / L_t} \qquad \text{Equation 1}$$

Where μ_{ep} is the analyte mobility, L_d is the effective column length, L_t is the total column length, t_m is the migration time of analyte and V is the applied voltage. This can be presented in Figure 1.4.

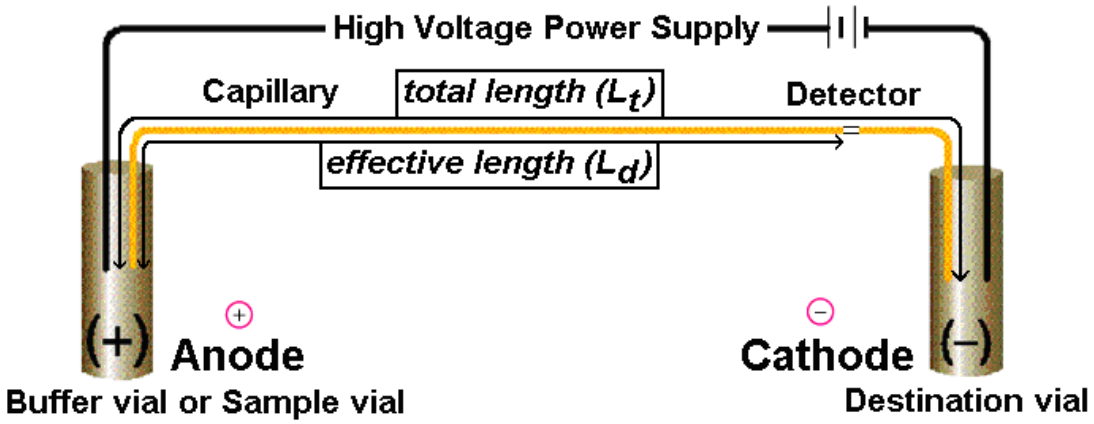


Figure 1.4 A scheme of total and effective capillary length

1.3 Chromatographic parameters

Several important parameters can be determined from the chromatogram in order to identify and quantify the components present and these are described in Figure 1.5 below. The chromatographic separation performance can be evaluated by the following parameters [15].

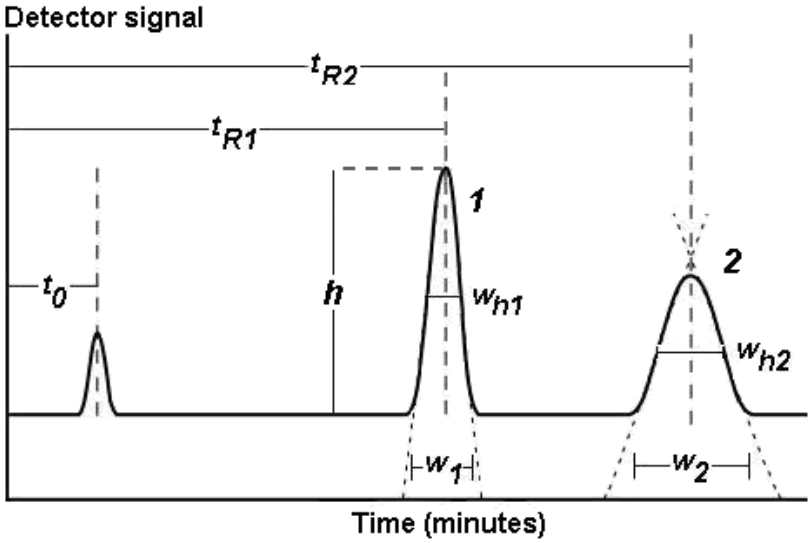


Figure 1.5 A Chromatogram showing void time, retention times, peak widths, peak widths at half height and peak height

Where t_0 is the void time

t_{R1} is the retention time of the first interested analyte

t_{R2} is the retention time of the second interested analyte

h is the peak height of the interested analyte

w_1 is the peak width at base line of the first analyte

w_2 is the peak width at base line of the second analyte

w_{h1} is the peak width at half height of the first analyte

w_{h2} is the peak width at half height of the second analyte

The chromatographic parameters which indicate the separation performance can be calculated as these following details.

1.3.1 The capacity factor or retention factor (k')

This calculation is to define the retention ability of a compound. The more value of the capacity factor, the more retention property of the compound in the column. This value can be determined using Equation 2.

$$k' = \frac{t_R - t_0}{t_0} \qquad \text{Equation 2}$$

Where t_R is the retention time of the interested analyte, and t_0 is the void time.

1.3.2 The selectivity (α)

This is a parameter which is used for a relative comparison of two compounds in the same chromatographic system. The value is normally more than one which means those two compounds have different retention properties and also shows their degree of separation. The selectivity can be calculated using Equation 3.

$$\alpha = k'_1 / k'_2 \qquad \text{Equation 3}$$

Where k'_1 and k'_2 are the capacity factors of the first and second analyte respectively.

1.3.3 The number of theoretical plates (N)

This is the factor to indicate the efficiency of the column. The number of theoretical plates shows an ideal value of the separation equilibrations in a column. The higher the value of *N*, the better the column efficiency. Equation 4 and Equation 5 show how to calculate *N* [16].

$$N = 5.54 \left[\frac{t_R}{w_h} \right]^2 \qquad \text{Equation 4}$$

$$N = 16 \left[\frac{t_R}{w} \right]^2 \qquad \text{Equation 5}$$

Where t_R is the retention time, w_h is the peak width at half height, and w is the peak width at baseline.

The Equation 4 will be used for calculation the column performance for this study.

1.3.4 The plate height (H) or height equivalent to a theoretical plate (HETP)

This value describes the height of each theoretical plate. The higher the value, the poorer the equilibration is obtained. The calculation of *H* is shown by Equation 6.

$$H = \frac{L}{N} \qquad \text{Equation 6}$$

Where *L* is the column length and *N* is the number of theoretical plates on the column.

1.3.5 Resolution (R_s)

This is the factor to define quantitative separation between two adjacent peaks. When the R_s value is 1.5, it indicates baseline separation. Ideally, the goal of most chromatographic methods is to achieve baseline separation (R_s = 1.5 – 2.0) for all key analytes [17]. The following Equations 7 and Equation 8 describe the calculation of R_s.

$$R_s = \frac{1.18\,(t_{R2} - t_{R1})}{w_{h1} + w_{h2}} \qquad \text{Equation 7} \quad \text{or}$$

$$R_s = \frac{2\,(t_{R2} - t_{R1})}{w_1 + w_2} \qquad \text{Equation 8}$$

Where t_{R1} and t_{R2} are the retention times of the analytes in order, w_{h1} and w_{h2} are their peak widths at the half-height, and w_1 and w_2 are their baseline peak width.

Also resolution can be presented in term of *k', α* and *N* using the following Equation 9 first described by Purnell [1].

$$R_S = \frac{1}{4}\left[\frac{k'}{(1+k')}\right](\alpha - 1)\sqrt{N}$$ Equation 9

1.3.6 Asymmetry factor or peak symmetry or tailing factor (A_S)

Under Ideal conditions chromatographic peaks should be symmetrical. However, in some instances, they exhibit either fronting or tailing. The most commonly used method to quantify peak symmetry is the asymmetry factor (A_S), typically calculated using the peak width at 10% of peak height as shown in Figure 1.6 and Equation 10.

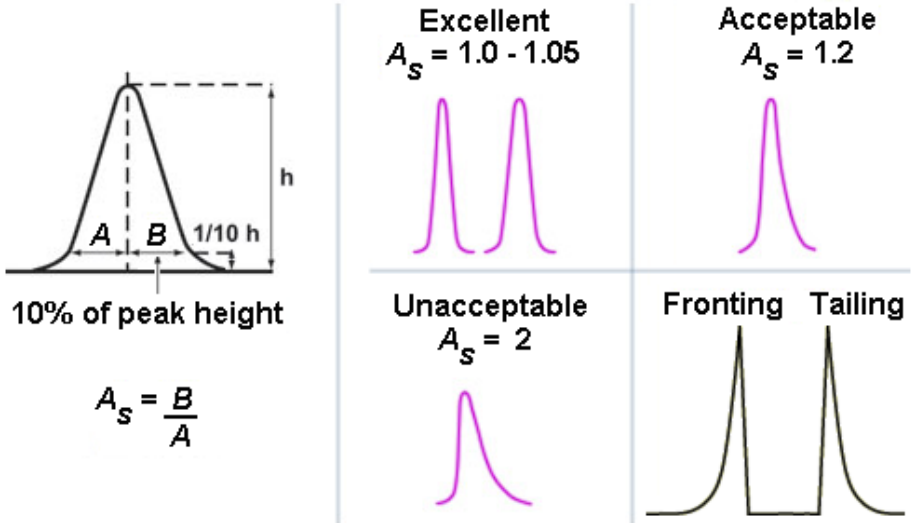


Figure 1.6 Illustration of peak symmetry and asymmetry factor calculation

$$As = B / A$$ Equation 10

Where *A* is the first half peak width at 10% of peak height and *B* is the second half peak width at 10% of peak height.

The acceptable values of *As* for chromatographic peaks are from 0.9 to 1.2.

Ideally, chromatographic peak has a Gaussian profile which is from a partitioning of analytes between stationary phase and mobile phase. An example of tailing (*As* > 1.2) can be explained that some analytes retain more strongly than others, while fronting (*As* < 0.9) is more often a result of sample overloading onto a column.

1.4 Theory of band broadening

Band broadening describes how the width of the narrow band on top of the column increases as the analyte migrates through a chromatographic system and becomes diluted in the mobile phase. The longer the analyte travels through a column, the greater the individual molecules spread out and the wider the band becomes thus producing broader chromatographic peaks with less efficiency. This band broadening can be described by three parameters first reported by J.J. van Deemter in 1956 [18]. Originally, it was introduced for gas chromatography, but it happened that the same physical process occurs in HPLC, and this equation is perfectly suitable for liquid chromatography. This can be shown in Equation 11.

$$H = A + \frac{B}{u} + Cu$$

Equation 11

Where *A* is Eddy diffusion, *B* is longitudinal diffusion and *C* is resistance to mass transfer in the stationary phase and mobile phase.

The van Deemter equation describes band broadening in chromatography due to the migration of an analyte through the column. This combines all sources and factors and represents them as the dependence of *HETP* on the mobile phase linear velocity (*u*):

These three parameters can be described in detail the band broadening is illustrated in Figure 1.7.

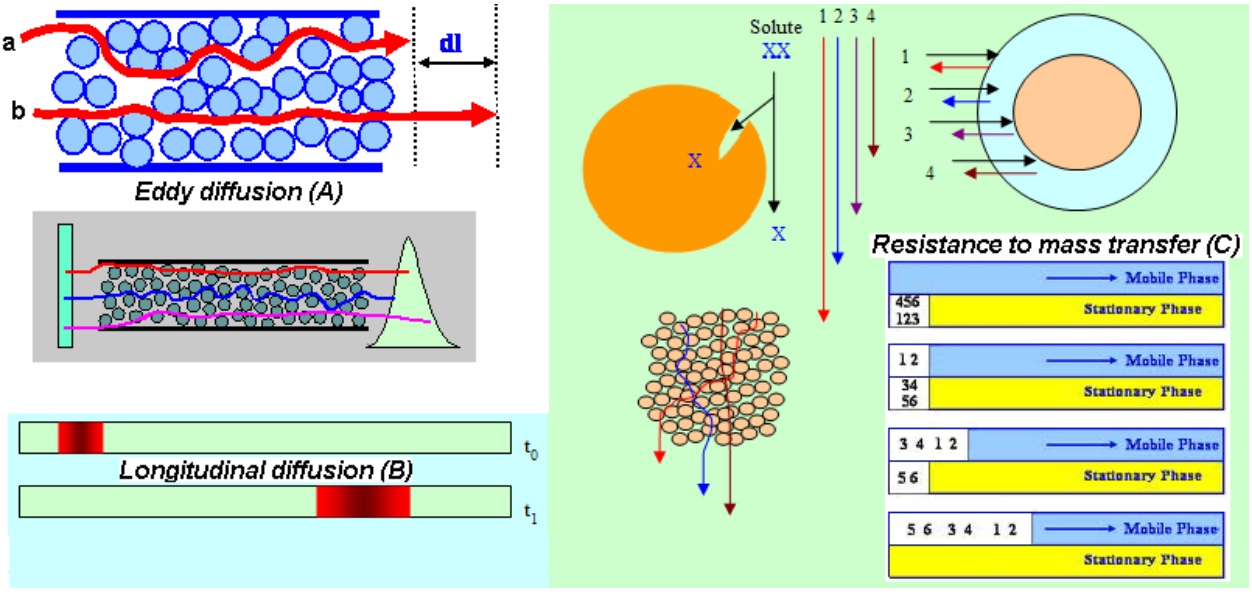


Figure 1.7 Three contributions of band broadening (modified from Introduction to LC 2012 [20])

1.4.1 Eddy diffusion (A)

This term explains multiple paths dispersion of individual analyte molecules as they travel differently through a chromatographic column. Some molecules will take longer paths while others, for example those closer to the walls of the column will travel faster. Therefore they will elute and reach the detector first. This is a velocity-independent term which is related to particle size and distribution and other non-uniformity in the packing bed. This term can be minimised by using well packed columns and uniform packing material.

1.4.2 Longitudinal diffusion (B)

This is a result of natural diffusion of solute molecules in the mobile phase along the column which can diffuse in all directions. However, the B term refers to diffusion in the axial direction only. The extent of this parameter depends on the time spent by the solute molecule in the column. Therefore the longer the solute remains in the column, the greater the extent of diffusion will be. In order to reduce this diffusion, the flow rate should be increased. However, the over increased flow rate will then bring the higher value of resistance to mass transfer (*C*).

1.4.3 Resistance to mass transfer (C)

This term is the most important factor affecting column efficiency and describes the partition of an analyte between the stationary phase and mobile phase. In contrast to longitudinal diffusion, increasing the flow rate causes solute molecules that reside in or on the stationary phase for varying lengths of time, such that when they are desorbed, they will have been left behind and the bulk of the molecules and the solute band will have broadened [4]. This factor can be further divided into the moving and stagnant mobile phase mass transfer. Moving mobile phase mass transfer is described as the result of molecules in the same flow path traveling at different speeds whereas stagnant mobile phase mass transfer is explained as the distribution rate of the solutes between the two phases. The molecules which are close to the column wall will move slower than those in the bulk of the stream. This moving mobile phase mass transfer will cause band broadening. In addition, an example of band broadening from stagnant mobile phase mass transfer is that

molecules which only diffuse a short distance into the pores will catch-up with the bulk mobile phase more quickly than those diffusing further into the pores. A typical van Deemter plot (*HETP* versus linear velocity, *u*) illustrates the point at which the sum of the three contributions produce a minimum value, giving the optimum linear velocity (u_{opt}) and flow rate of the mobile phase (Figure 1.8). The optimum flow rate is chosen where the *HETP* is lowest (H_{min}), and hence the column efficiency is highest.

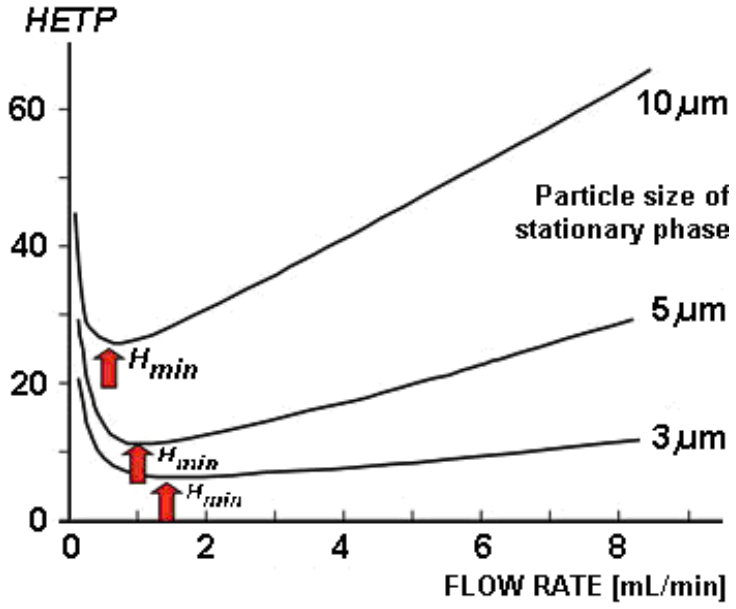


Figure 1.8 A typical van Deemter plot showing contributions from the three dispersion factors (modified from Dong [17])

The linear velocity, *u* can be calculated using:

$$F = u \pi r^2 \qquad \text{Equation 12}$$

Where F is volumetric flow rate, *u* is linear velocity, and r is radius of the column

1.5 Monolithic materials and monolithic columns

1.5.1 Monolithic materials

Monoliths are a fabrication of polymeric materials which contain numerous interconnected channels [20]. This can introduce a high permeability to the monolith. Monoliths can be fabricated to have variable porosity and consequently varying surface area [21]. They have been developed to overcome the drawbacks of packed capillary columns. For example, classical packed capillary columns usually face problems with frit properties/fabrication, while monolithic stationary phases overcome this by using anchoring sites on the capillary surface. In addition, the monoliths can easily be prepared or modified using simple polymerisation and chemical reactions.

Monoliths are divided into two types, inorganic and organic. Silica monoliths or sol-gel monoliths are for example prepared using alkoxysilane polycondensation reactions whereas the organic monolith is a co-polymer fabricated using free radical polymerisation [22]. The morphology and pore size of monolith are resulted from type and structure of monomers and porogenic solvents. During the polymerisation, the separation phase occurs and governs the pore formation structure for the monolith. The pore property will be caused by a solvation of porogenic solvents regarding their content in the mixture also. A good solvation of porogen will form small pores (mesopores), on the other hand a bad solvation of porogen will provide macropores [23].

To study the characteristic of this type of monolith, free radical polymerisation will be used, which involves three kinetic processes, namely initiation, propagation and termination [24], [25]. The initiation process employs free radicals from the initiator which continuously induce a free radical formation of monomer and cross-linker molecules. Rapid free radical reactions occur simultaneously then the new covalent bond between two free radical molecules is created, chains of polymer are formed in the propagation step respectively. The termination step occurs when the polymer chain formation is closed at the end of the polymer chain.

Organic types of monolith are prepared from a mixture including a functional monomer, a cross-linker, a porogenic solvent, and an initiator.

1.5.1.1 Functional monomers

The monomer containing the desired functional group will influence the characteristic of a stationary phase of a monolith. In 2007, Vlakh and Tennikova [21] reported that the higher percentage of monomer in the polymerisation mixture, the lower the permeability of the final monolithic material. Methacrylates are common examples of functional monomers. The chemical structures of the organic functional monomers SPE, and PEDAS are shown in Figure 1.9.

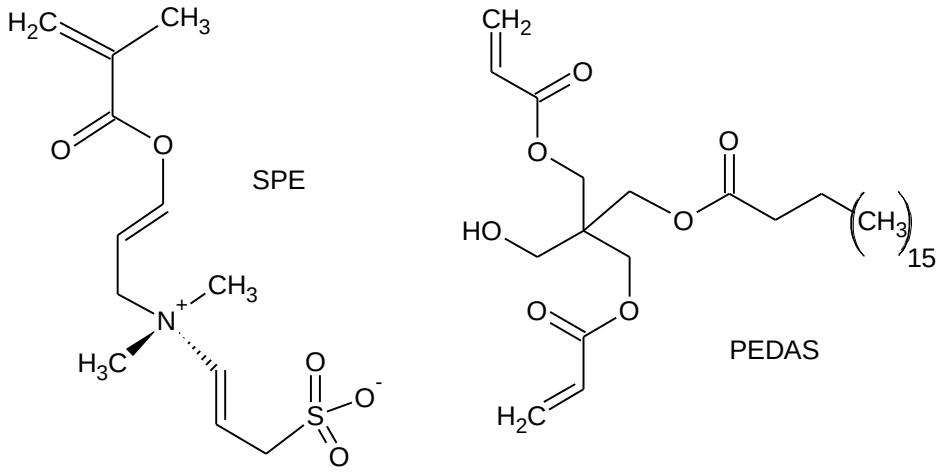


Figure 1.9 Chemical structures of organic functional monomers SPE, and PEDAS

1.5.1.2 Cross-linkers

A cross-linker is basically a monomer that can build a polymer by attaching functional monomers together. Good cross linkers are those which readily form free radicals. Rigidity, homogeneity and porosity of a monolith are influenced by the proportion of cross-linker in the mixture [21]. Some examples of cross-linkers are ethylene glycol dimethacrylate (EDMA) and pentaerythritol diacrylate monostearate (PEDAS). EDMA has two polymerisable vinyl groups at both ends, whilst PEDAS not only has a long alkyl chain group which supplies a hydrophobic property, but also has self polymerisable vinyl groups on both ends which can react with another adjacent PEDAS molecule. Thus PEDAS can be used both as a functional monomer and a cross-linker in monolithic preparation [21]. Figure 1.10 shows examples of the chemical structure of cross-linkers EDMA and PEDAS.

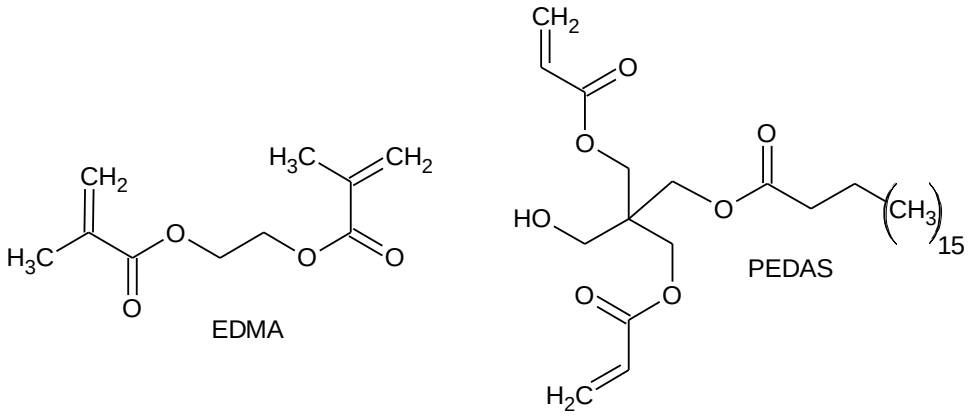


Figure 1.10 Chemical structures of cross-linkers EDMA, and PEDAS

1.5.1.3 Porogenic solvents

A porogenic solvent or a porogen is a solvent or co-solvent used for monolith polymerisation reactions and directly provides the porosity of the monolithic stationary phase. The type and proportion in the organic phase of porogenic solvent is a powerful factor in building the desired porous structure of the monolith [21]. Porogens can be divided into 2 groups such as macro-porogens (those that create through pores) and mesoporogens (those that create mesopores) reckoning on the size of pores they can form in the polymer structure. The solubility of the polymer in a porogen affects its porosity which is attributed to the time of phase separation of compounds. The higher the solubility of the system, the longer time for phase separation because of the formation of small micro-globules. Poor efficiency of the column will occur if large pores are formed as a result of having a poorly soluble system. Finally, the proportion of porogen optimisation is essential in order to provide the desired characteristic properties of the monolith in terms of permeability and chromatographic performance [21]. Some examples of common

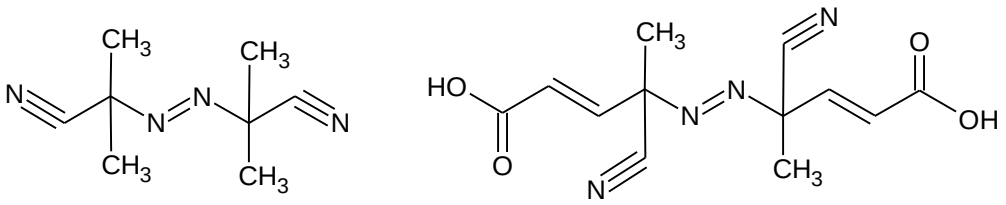
porogenic solvents are methanol, cyclohexanol, decanol, dodecanol, N-methylpyrrolidone, and 1, 1, 1, 2 - tetrafluorethane [26].

1.5.1.4 Initiators

This is the essential compound to make the reaction start. Basically it is employed to generate free radicals and then transfer these to other compounds which then make monolith polymerisation commence. There are four kinds of initiation process, namely thermal initiation, photo initiation, gamma radiation and finally microwave radiation as described below.

1.5.1.4.1 Thermal initiation

This method uses a chemical initiator such as aza compounds for example 2, 2-azobisisobutyronitrile (AIBN) or 4, 4'-azobis (4-cyanopentanoic acid) (V-501) as shown in Figure 1.11 [27].



AIBN V-501

Figure 1.11 Chemical structures of initiator AIBN, and V-501

This is the most common method used for initiation only requiring a column oven to perform the reaction. The rate of reaction will be controlled by the proportion of initiator, length of time for polymerisation and temperature of the system. This method usually takes at least 12 hours to complete the reaction [21].

1.5.1.4.2 Photo initiation

This method uses photolysis to complete the reaction via a source of ultraviolet radiation (UV).In order to be effective a transparent capillary will be needed and the procedure usually occurs at room temperature. In 2005, Khimich et. al [28] investigated the preparation of poly glycidyl methacrylate (GMA) fabricated with ethylene glycol dimethacrylate (EDMA) using photo initiated polymerisation with AIBN as initiator and cyclohexanol as a porogen. The factors affecting photo initiation are intensity and wavelength of the light source including nature and concentration of the initiator. Some widely used photo initiators are 2-methoxy-2-phenylacetophenone, 2, 2-dimethoxy-2-phenylacetophenone (DMPA) and AIBN.

1.5.1.4.3 Gamma radiation

In this method, there is no initiator required to generate free radicals but gamma radiation will be homogeneously carried out instead within minutes. In 2005, Safranyl et. al. [29] studied some porous structure of monolithic preparation using a gamma radiation initiated polymerisation. However this method has not been used regularly because of the lack of availability of suitable instruments and safety concerns [21].

1.5.1.4.4 Microwave radiation

This method can save a lot of reaction time compared to other radiation methods with the power output and time being control factors. Recently, a study was carried out by Whungsinsujarit [30] on monolith preparation using a microwave radiation

technique which showed that, the efficiency of columns produced in this way still produced uncertain results.

1.5.2 Monolithic columns

Monoliths were first discovered and successfully applied for liquid chromatography in the early 1990's by Hjerten [31] and Nakanishi and Soga [32]. They were used as a replacement for particle packed columns, giving high permeability with a good separation and efficiency.

Monoliths can be manufactured using in-situ polymerisation of a monomers mixture solution and reacted successfully to the inner walls of capillary. These chemical bonds can eliminate the need of having frits in capillary columns and also can retain the polymers in columns when applying high pressure of DiNa-pump. Basically the performance of particle packed columns is evaluated and determined using their chemical structures, in particular the performance of monolithic columns (silica or organic) is determined by their pore structures and their morphologies instead. These are affected by factors involved in their chemical fabrications, such as nature of monomers, porogen and initiator in polymerisation conditions. The work of Tallerek et. al. [33], [34] using CLSM characterisation demonstrated the importance of these factors. Therefore, monoliths and their optimisation conditions will be studied and discussed in further sections.

1.5.2.1 Silica-based monolithic columns

Silica monoliths have been successfully used for separations of both small and large molecules over the last 20 years [35]. A spongy characteristic with round

pores and a network skeleton structure are formed in silica monoliths [33]. Basically, a surface chemistry of silica monoliths is similar to particle packed columns. A larger through-pore/skeleton size ratio (1.2-2.5) than a particle packed columns (0.25-0.4) was found [36], [37]. There was 65% external porosity in silica monolith columns whereas only 25% external porosity was found in particle packed columns [38]. As a consequence, they provided shorter diffusion path length in the stationary phase and lower flow resistance, simultaneously.

1.5.2.1.1 Preparation of silica monoliths

Silica monoliths are prepared by hydrolyzing a mixture of silane compounds in the presence of an inert compound and the porogen. The spinodal decomposition is exhibited called sol preparation and hydrolysis. It can increase to periodic domains (silica-rich and solvent-rich). Then these formed structures are frozen by gelation (washing and aging of the gel), giving the strength polymeric skeleton structure with through-pores and mesopores [39]. Unreacted monomer and porogens after polymerisation are washed from the column with an appropriate solvent. Finally, the silica monoliths may be modified with desired surface chemistry reagents using general thermal initiation which is the most popular method or photo initiation [40], [41].

1.5.2.1.2 Silica monolith structure

The skeletal network of silica monoliths has been explained as aggregated silica particles varying in size and through-pore distributions affected by the above mentioned factors. The permeability is oppositely associated with the domain size, corresponding to that in particle packed columns. However, the overall permeability is higher for monolithic columns. Nakanishi and Soga [32], [43], [44], [45] studied and developed silica monolithic structures in capillary columns varying affected factors including size of bed, polymerisation factors for example different porogens, pH of wash liquid, and temperature of polymerisation governing the better chromatographic efficiency.

1.5.2.1.3 Performance of silica monoliths

Factors to improve the separation efficiencies of monolithic phases are smaller domain size, high phase ratio (volume of mobile phase to stationary phase), and good bed homogeneity [46]. The similar minimum plate height value of monolithic and silica packed columns was reported by Kobayashi et. Al. [47]. However increasing mobile phase velocity gave much less efficiency of silica monolithic columns than packed columns. This can be explained by some parameters of the van Deemter equation, a larger A coefficient and a smaller C coefficient for monolithic columns compared to particle packed columns provided a less efficiency. Many researchers discovered and studied factors affecting silica monolithic columns and particle packed columns improving efficiencies [38], [45], [48], [49], [50], [51], [52], [53],. Marisato et. al. [48] studied a kinetic plot analysis of

silica monoliths and particle packed columns. Minakuchi et. al. [39] discovered macropore diameter and skeleton thickness of monolithic columns at 1 μm performed equivalent to a 3 μm particle column in column performance. In the similar results from Minakuchi et. al. [39], silica monoliths with smaller size skeletons revealed van Deemter plots (amylbenzene and insulin) with minimum plate heights at higher linear mobile phase velocities than for particle packed columns. The smaller skeleton size decreased the slope of the curve which was because of the short diffusion path length associated with the smaller skeleton size (less contribution to the plate height C term). Minakuchi et. al. [49] found the better efficiency, less plate height in the monolithic columns with a reduction in domain size and also reported a smaller effect of mobile phase linear velocity on plate height for amylbenzenes. Diffusion in mesopores is slower for large molecules such as insulin, a greater influence in the C term of the van Deemter equation is exhibited. In the same study, the optimization of domain size for best column efficiency was studied, but found that the lower performance actually achieved than that expected [49]. The A coefficient increased and the C coefficient decreased for van Deemter plots with a decrease in domain size indicated that the mobile phase mass transfer was slower although the small domain size expedited faster mass transfer in the stationary phase [50]. Desmet et. al. [51], [52] revealed greater irregularity in monoliths with small skeleton size and wider through-pore size distribution facilitating in worse performance than expected and also reported poor resolution in separation using these silica monoliths because of having a smaller phase ratio. In the same study they also presented theoretically that the

performance of silica monoliths with small domain size can be greatly improved by increasing the homogeneity of the skeleton and through-pores with increasing the phase ratio. Hara et. al. [46] successfully manufactured and modified silica monoliths (stationary phase bed structure) with high phase ratio, small domain size and homogenous skeleton then an optimisation of chromatographic parameters can improve particle packed column performance. A decrease in the plate height for insulin with an increase in temperature affected the lower value of the A and C terms to the van Deemter equation reported by Leinweber et. al. [53]. This can be explained because the higher temperature, the higher both the lateral mass transfer and the intra-skeleton mass transfer. Desmet et. al. [54] also used kinetic plots to describe the better performance of silica monolith column than that particle packed column. In the study they also suggested synthethic methods to improve the bed structure homogeneity and decrease the domain size of monoliths to generate the best column performance.

1.5.2.2 Organic polymer based monoliths

Organic monoliths were first successfully promoted and exploited in the 1989 by Hjertén [31]. The manufacturing of a highly swollen cross-linked gel of *N, N*-methylenebisacrylamide and acrylic acid in a salt forms in an aqueous medium was first introduced. Organic monolith materials are classified in three main groups including styrene based, methacrylate based, and acrylamide based structures. Since 1989, organic monoliths have been greatly modified to improve better performance for large molecule separations than silica monoliths because of their

biocompatibility and large domain size (cauliflower-like) structure, as can be seen in Figure 1.12 [20], [55].

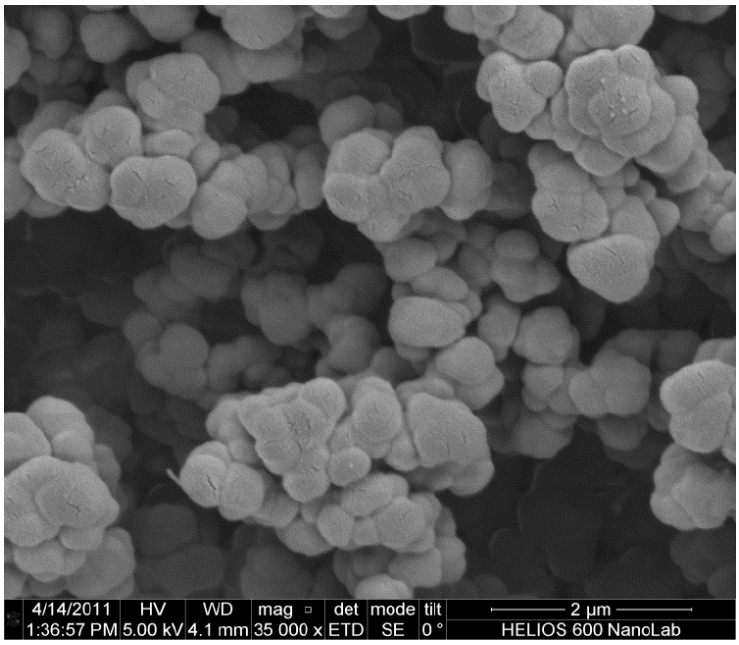


Figure 1.12 SEM of (cauliflower-like) morphology of organic monoliths (modified from Tennikova et. al. 2009)

Basically, the performance of polymeric monolithic column in the isocratic separation mode for small molecular weight organic compounds is relatively poor [56]. This might be explained by the absence of mesopores or the presence of micropores in the bed structures of the monoliths and also structural inhomogeneity providing the flow dispersion and band broadening [35], [57]. The poor performance to heterogeneous gel porosity in the globular structure of the organic monolith was reported by Nischang et. al. [58], generating from a radial distribution of the cross-linker density in the globule. Correspondingly, it boosted band expanding for retained compounds and gradually diminished the separation,

and results in a totally unsuitable material for small molecule separation. There are many literature reviews reporting organic monolith synthesis methods and performance, but with little underlining on bed structure [55], [58], [59].

1.5.2.2.1 Preparation of organic monoliths

There are 2 important steps concerning in organic monoliths preparation in capillary columns for example inner surface modification and initiation of polymerisation of polymers in polymer mixture. First, silanisation is occurred from silanol groups of the capillary inner wall reacting with a bi-functional reagent. Second, polymerisation reaction is formed in the capillary by filling with a polymer mixture comprised of initiator, monomers and porogens, and sealed both ends with rubber septum, followed by thermal, microwave or photo-initiated polymerisation. In the time of polymerisation, the strong covalent bonds are formed between the capillary surface and monoliths to secure that the monoliths can tolerate relatively high pressure without being evicted from the capillary when using the DiNa-pump.

1.5.2.2.2 Modification of the capillary surface

There are many bifunctional silanising agents such as vinyl silane, acrylate silane or methacrylate silane to use for modification. The most common reagent modified is 3-(trimethoxysilyl) propyl methacrylate (γ-MAPs) [60]. Basically, capillary surface modification employs capillary pretreatment, silanisation, cleaning and drying procedures which are explained in the further experiment. Many researchers including Courtois et. al. [60], Vidic et. al. [61], Cifuentes et. al. [62] optimised and developed the pretreatment and silanisation procedures regarded surface

modification. The optimised protocol for this step will be a guideline for our research and will be used in further experiment.

1.5.2.2.3 Monolith polymerisation

After finishing the surface modification, the γ-MAPs ready capillary is filled with a prepolymer mixture and further fabricated using thermal, microwave or UV initiation methods. The monomers may include a functional monomer with a cross-linker offering a desired chemical property of monoliths. A low or high molecular weight inert solvent can be used as a porogen devolving on generating pores in the monolith. The porogenic solvents provide different thermodynamic properties which lead differential phase separations after polymerisation. Functional monomers, cross-linkers, porogenic solvents, and the initiation method notably affect the polymerisation mechanism and phase separation. Therefore these factors can greatly influence monolith morphology, pore size distribution and separation performance. These are needed to be further studied and optimised to get our in-house protocol for the research.

1.5.2.2.4 Organic monolith structure

Regarding bed structure morphology and porosity of organic monoliths precisely influence the column performance similar to particle packed columns and silica monolithic column. Organic monolithic structure should provide both large surface area and high permeability. A large surface area offers more active sites for better and effective interactions between analytes and monoliths. High permeability concedes faster determination and compromising backpressure. Moreover,

porosity is considerably most important morphology characteristic because it impinges the size and shape of both microglobules and clusters in monoliths. Therefore, the factors affecting organic monolithic structure should be optimised for their performance.

1.5.2.2.5 Effect of initiation method, porogen and initiator

Temperature, light intensity, microwave frequency intensity, and time of reaction are various factors affecting the rate of monolith polymerisation reaction which eventually governs the monolith morphology. These parameters undertake the initiation method which can be radiation polymerisation [63], living polymerisation [64], high internal phase emulsion polymerisation (HIPE) [65] and polycondensation [66].

Svec [67] reported different initiation methods employed different monolith morphologies; for example HIPE [65] gave an open pore monolith whereas thermal or photo initiation method provided globular or fused morphology. Thermal and photo initiation are commonly used for initiation method of organic monolith synthesis. The thermal initiation was successfully documented by Svec and Frechet [68] using a porous poly (glycidyl methacrylate-coethylene dimethacrylate) monolith with 1% 2, 2'-azobisisobutyronitrile (AIBN) as the thermal initiator. The effects of polymerisation temperature, polymerisation time, and type and concentration of thermal initiator on the morphology of the monoliths were also optimised [69]. Viklund et. al. [70] presented the pore size distribution of organic monoliths value gradually decreased when increasing polymerisation temperature

and subsequently increasing in surface area. They appointed the cause of higher decomposition rate of initiator and subsequently, polymerisation rate. Increasing of temperature also led to higher solubility of the monomer, therefore resulting in prolong separation and large pore size. However, the decomposition rate is much more influence than temperature. The porosity of organic monolith can be changed and optimised by varying polymerisation time. Svec et. al. [71] discovered the vanishing of large pores upon prolonged polymerisation, which were otherwise structured of the organic monolith in the initiation step of fabrication. However, Trojer et. al. [72] reported the significant increase in mesopore fraction when decreased in polymerisation time. They measured a surface area of monoliths and found significant increasing the surface area from 26.8 m_2/g to 77.2 m_2/g on decreasing the polymerisation time from 24 hours to 45 minutes. This was happened because of less cross-linking with shorter polymerisation time. These findings were also agreed from Nischang et. al. [56] who published a decrease in column performance with increase in polymerisation time. They explained this to enhance of resistance to mass transfer beginning from stagnant mass transfer zones in the porous monoliths. However the maximum rigidity of monolith requires sufficient polymerisation time, it is not extensively optimised to suit the pore size distribution. Organic monolith morphology and porosity are influenced from initiator type and concentration. A large number of free radicals is governed from smaller microglobules producing by a higher concentration of initiator [73]. Many researches on development of morphology and porosity of organic monoliths were found [28], [73], [74], [75]. They reported and published effects of porogenic

solvents using on organic monoliths. Volatile organic solvents such as ethyl ether, methanol and hexanes are commonly used as porogens [73]. Khimich et. al. [28] reported the effect of initiator concentration that an increase from 0.2 to 1% could increase the polymer density and formation of pore uniformity of monolith. In another research, Viklund et. al. [74] documented a breakage in continuous polymer structure was found when using approximately 3-4% concentration of initiator. The effect of temperature on photo polymerisation was studied in the research by Buszewski and Szumski [75], but it has been found to be less significant. Nowadays, both photo initiation and thermal initiation are still proportionately used for monolith polymerisation and both influence the organic monolith morphology.

The porosity of organic monolithic structure is altered by adjusting the natures of the porogenic solvents and/or their ratios regarding monomers concentration without changing the composition of the desired polymer [76]. The pore property of monolith is characterised by governing the solubility of the growing polymer chains in the polymerisation mixture and promoting differential phase separation in the uniform pre-polymer solution during fabrication reaction [77]. Basically, a poor porogenic solvent will give larger through pores by assisting prior beginning of phase separation. The new phase swells between the monomers because of the better thermodynamic of solvents for the polymer than the porogen. As a result, the large globules with larger voids between monomers are formed. On the other hand, smaller pores are formed by a good porogen by retarding the period of phase separation and attempting to the monomer in nuclei solvation. Many

researches on the effect of porogen on porosity have been well studied. Viklund et. al. [70] reported the increase in the pore size diameter of a poly (glycidyl methacrylate-co-ethylenedimethacrylate) monolith (GMA-EDMA) when adding a poor solvent (dodecanol). The pore size distribution of the monoliths increases from 150 nm to 2,570 nm when increasing the percentage of dodecanol from 0% to 15%. In contrast, an impressive decrease in pore size for a poly (styrene-co-divinylbenzene) monolith when adding small percentage of good solvent (toluene). Moreover, Santora et. al. [77] also reported the effect of non-polar porogen (*n*-hexane) on non-polar non-polar divinylbenzene-styrene (DVB/STY) monolith morphology and surface area. They found the non-polar porogen (*n*-hexane) productively formed high surface area while the polar porogen (methanol) generated smaller surface area. They also discovered the opposite that in a more polar ethylene dimethacrylate-methyl methacrylate (EDMA/MMA) monomer mixture. Hexane will generate low surface area while methanol will give high surface area structures instead. These monoliths had surface area as large as 820 m^2/g but contained rather small pores. Therefore, it is problematic when applying to the analysis system. Premstaller et. al. [78] presented a porogenic mixture of decanol and THF for a poly (styrene/divinylbenzene) monolith providing large through-pores and morphology without micropores (similar to nonporous particles). The authors successfully applied these columns to an analysis of oligonucleotides with high resolution and rapid separation. In addition, the ratio of porogens can also characterise the monolith morphology. Li et. al. [79] favorably used toluene and decanol as porogens to fabricate poly (bisphenol A dimethacrylate) (BADMA)

monolithic columns. They found the ratio of these porogens was very sensitive on porosity forming for these monoliths. They also reported the segregation and detachment of monoliths from the capillary wall when replacing of toluene with THF (good solvent). The facts that monoliths with low back pressure had larger microglobules and microglobule clusters and monoliths with high back pressure had much smaller size of microglobules were also discovered by them. Courtois et. al. [80] reported that the larger the molecular weight of poly (ethyleneglycol) (PEG) dissolved in 2-methoxyethanol using for fabrication of glycidyl methacrylate-cotrimethylolpropane trimethacrylate-co-triethylene glycol dimethacrylate) monoliths, the larger pores generated. Aggrawal et. al. [81] employed PPG-PEG-PPG triblock copolymers and diethyl ether as porogens to fabricate poly (ethylene glycol methyl ether acrylate-co-polyethylene glycol diacrylate) monoliths. They found an extensive amount of mesopores in the monomer structures. Aoki et. al. [82] used a combination of high molecular weight polystyrene (PS) and chlorobenzene to produce poly (glycerol dimethacrylate), (poly-GDMA) monoliths and found an interesting morphology. The effect of using poor porogenic solvent, toluene on preparation of the monoliths in situ showed a typical agglomerated globular structure while using the combination porogens led a transformation of morphology from an aggregated globule form to a continuous skeleton structure. These morphological changing can be explained with the pore size distribution showed a sharp bimodal distribution. The first peak was at around 4 nm in the mesopore range (2-50 nm) and the second peak located around 1-2 μm in the macropore range (>50 nm). Cooper et. al. [83], and Hebb et. al. [84] used atypical

porogen, supercritical carbon dioxide to prepare the poly EDMA-co-TRIM monoliths governing a wide range of through-pore diameters from 20 nm to 8 µm. The authors also discovered a direct effect of CO_2 pressure on monolith properties such as pore size, pore volume, and surface area. However, they need a special equipment to apply high pressures at about 15-30 MPa for the polymerisation. A selection of suitable porogens for preparing organic monoliths is still not elementary and it is achieved by experimentation. The published work of other researchers and their experience in selection of appropriate porogens can be a guideline for our study.

1.5.2.2.6 Effect of monomers

The chemical properties of a monomer or amount of a monomer in the fabrication procedure can influence the morphology and porosity of the organic monolith. The amount of cross-linker performs the globule size and morphology of monoliths. Similar to the using of a poor solvent, a using of higher concentration cross-linker provides early phase separation. Not only a single monomer can influence the polymerisation kinetics and the monolith morphology but it can also change the surface chemistry and separation selectivity. Smirnov et. al. [85] published an impressive reduction of the globules size, therefore the size of the pores between these globules was increased from 4% to 8% in weight fraction when using 2-hydroxyethyl methacrylate (HEMA) monomer in the monolith mixture. They increased the number of hydroxyl groups to enhance polymer and porogen interactions. Coincident results were found for monomer mixtures such as GMA/EDMA [71] and PS/DVB [86]. Santora et. al. [77] also published a reduction

of surface area when decreasing in monomer and cross-linker ratio in the fabrication mixture. Xu et. al. [87] reported the influence of changing in length and branching ratio of the cross-linker on column performance by setting the molar ratio of the cross-linker and the monomer fixed. The authors discovered an increase in the volume of small mesopores when increasing in the length of the cross-linker. Therefore, this can be resulted in better separation efficiency for small molecules. A small alkylbenzenes analysis was demonstrated in their research because of highly interconnected mesopores which led increasing in surface area and fast transfer kinetics for small molecules. Hence, the effective thickness of the diffusion layer in monolith was significantly decreased. Many researchers employed a single cross-linker to effectively increase the surface area and the concentration of needed mesopores in desired monolith. Aggrawal et. al. successfully fabricated several monoliths from single cross-linkers for example bisphenol A dimethacrylate, bisphenol A ethoxylate diacrylate (BAEDA, EO/phenol = 2 or 4) and pentaerythritol diacrylate monostearate (PDAM) [76]. They found differences in their morphologies eventhough using the same group of monomer (BAEDA-4, and BAEDA-2). Fused skeleton structures were formed instead of microglobules in monoliths. The high resolution separations of alkyl benzenes and alkyl parabens were established by these columns because of enhancing in surface area from the highly cross-linked structure. Urban et. al. [88] published small molecule separation using a mixture of styrene, vinylbenzyl chloride, and divinylbenzene monomers with cross-linking of the functional groups on the surface by Friedel-Crafts alkylation. They found a significant growth of surface

area of the monolith and a symbolic increase in the concentration of mesopores following hypercross-linking.

1.5.2.2.7 Effect of monomer to porogen ratio

Trojer et. al. [89], and Tolley et. Al. [90] discovered the influence of monomer concentration on the properties for poly [p-methylstyrene-co-1, 2-(p-vinylphenyl) ethane] monoliths. They found a decrease in macropore distribution size from 8.78 to 0.09 μm when increasing in the total monomer to porogen ratio from 35% to 45% (v/v). This is because a larger number of nuclei formed via the reaction of higher concentrated monomers. Higher density of nuclei compare with the monomer, their sizes propagate much slower before they react to each other. Smaller macropores are therefore formed between the microglobules in clusters for the final polymeric monolith. Consequently, in most cases of a tolerable solvent flow on the operating pressure limits of LC instrument, the monomer to porogen ratio should not be higher than 50%. Not only a decrease in the monomer concentration generates larger macropores but it abates the density and rigidity of the monolith also. It was found that the fabrication of trimethylolpropane trimethacrylate (TRIM) monolithic polymers was not occurred when using low monomer concentration (< 0.5 g/mL), but instead produced in a powder [92]. Li et. al. [93] also found a decrease in rigidity because of using a lower initial monomer concentration for a synthesis of poly (triethylene glycol dimethacrylate) monoliths. The concentration of a 32.2% monomer by weight for preparation of monoliths should be kept in dry condition but when concentration decreasing to 20.2% by weight, monolith will provide lower back pressure and will be deteriorated after

drying. Smirnov et. al. [85] actively demonstrated an effect of increase in monomer concentration in a polymerisation mixture on an increase in column permeability. Eeltink et. al. [91] successfully fabricated low density methacrylate monoliths (20% monomer content) containing a high porosity structure. Consequently, the column efficiency was evaluated comparison between low-density monoliths and high-density monoliths.

1.5.2.2.8 Performance of organic monoliths

Similar to those any other stationary phases, the important chromatographic performance parameters for example efficiency, resolution and permeability of organic monolithic columns result from the pore size distribution and skeletal structure. Organic monoliths have been mainly applied for large biomolecule separations whereas silica monoliths have been primarily used for both small and large molecules regarding globular skeleton found in their morphologies [92]. Recently, Nischang et. al. [56] and Li et. al. [90] successfully published separations of small molecules using organic monoliths.

1.5.2.2.9 Effect of initiation method

The monolith morphology has been modified from an initiation method, length of time and condition of polymerisation. Trojer et. al. [72] and Nischang et. al. [56] presented shorter polymerisation time to be optimised and supportive for small molecule separation because of a result in increasing mesopore volume fragment. For the van Deemter equation, decreasing in the resistance to mass transfer and eddy diffusion term contributions influences from a decrease in though-pore size of

monoliths. This occurs when increasing polymerisation temperature and finally affects an increase in the column performance.

1.5.2.2.10 Effect of porogens

As previous description in Section 1.6.2.2, the porogens govern the porosity and a pore size distribution of the monoliths. Changing the type or the content of porogen concludes the application of monolith for small or large molecule separations and also influences the column performance for an appropriate separation. The 40% better performance than particle packed columns of an organic monolithic column with micropellicular morphology for oligodeoxynucleotide separation was successfully exhibited by Premstaller et. al. [78]. This was referred to a decrease in intraparticle dispersion because of a total deficiency of small pores in the monolithic structure, conceding only effective flow through the skeleton structure. In contrast, Li et. al. [79] demonstrated using poly (BADMA) monolithic columns with small microglobules or fused morphologies for separation of small molecules such as alkylbenzenes and alkylparabens. They reported a column performance in term of the theoretical plate numbers between 20,000 and 30,000 plates/m for uracil at 0.1 µL/min (i.e., 0.38 mm/s) and 61,432 plates/m for retained compounds. High surface area and small size of organic monolithic structure assigns high performance of the column. Aoki et. al. [82] successfully reported the situ monolithic column efficiency with high molecular weight polystyrene as coporogen at 34,075 plates/m (H = 29.3 µm) which was much higher than 5,650 plates/m (H = 177.0 µm), and 1,335 plates/m (H = 749.3 µm) using toluene, or low molecular weight polystyrene (PS) as coporogen. This is because the high molecular weight

PS porogen prolonged phase separation due to viscoelasticity. Sized exclusion chromatography using poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide) (PEO-PPO-PEO) or PPOPEO-PPO organic monoliths with Brij 58P as mesoporogen were successfully fabricated by Li. et. al. [93], and Bakry et. al. [94]. They found the mesopores in the monolithic structure which governed the good separation.

1.5.2.2.11 Effect of monomers and monomer to porogen ratio

Smirnov et. al. [85] discovered the notable increase in column efficiency of HEMA monoliths when increasing from 4% to 8% monomer content. They found the plate height decreased from 188 to 51 μm for a non-retained compound. This is because of a reduction of globule size in the monolithic structure. Xu et. al. [87] also published an eight times increase in number of theoretical plates/m (from 11,000 to 83,000 for thiourea) when altering ethylene dimethacrylate (EDMA) to 2-methyl-1, 8-octanediol dimethacrylate (2-Me-1, 8-ODDMA) as a cross-linker. The reduction of C term in the van Deemter equation was a result from an increase in mesopores fraction when using 2-Me-1, 8-ODDMA cross-linker. Urban et. al. [88] successfully manufactured hypercross-linked columns showing a plate height (H) value of 39 μm for benzene. A rapid isocratic elution of peptides and a gradient separation of 7 small molecules using same column were demonstrated by them. They also reported this column to analyse polystyrene standards using an organic mobile phase by size exclusion chromatography.

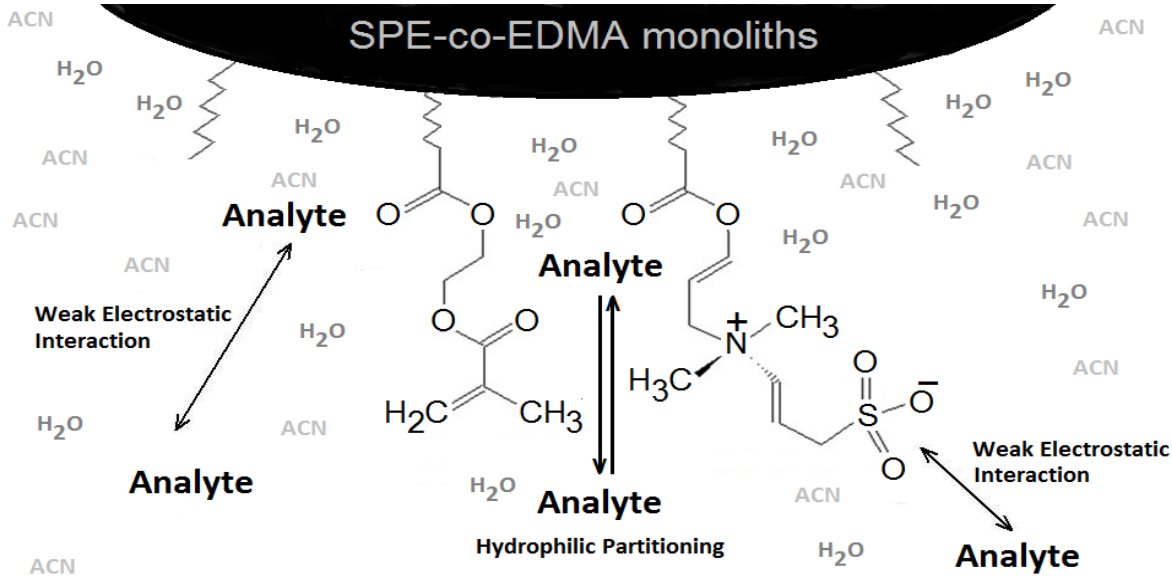
Eeltink et. al. [91] established a 5 times increase in column efficiency for a small molecule due to an increase in the porosity when decreasing the monomers from 40% to 20% content. In the research of Trojer et. al. [89], the separation resolution increased whereas the retention times for biomolecules were consistent by increasing the monomer content. In the study, a change in monomer to porogen ratio influenced in both mesopore fraction and through-pore size of monolith altering both the retention times and resolution of oligonucleotides analysis. Due to an increase in surface area, the small molecule interaction with the stationary phase increases influencing an increase in pore size distribution which is needed for a separation of small molecules.

1.6 Hydrophilic Interaction Liquid Chromatography (HILIC)

Hydrophilic Interaction Liquid Chromatography (HILIC) is an alternative mode to separate polar and hydrophilic compounds, which has also recently seen an increase in applications in the past decade. The mechanism was first proposed by Alpert in 1990 [95] HILIC employs normal phase retention, as it contains a polar stationary phase but uses a similar mobile phase to reversed-phase high-performance liquid chromatography (HPLC). Therefore with increasing analyte polarity, the greater the retention, allowing for more efficient separation. Typical mobile phases used are composed of a high percentage of acetonitrile (ACN) and a small percentage of aqueous buffer, for example 90: 10 % v/v ACN: aqueous buffer. By using this mobile phase two layers are formed with different polarities, a water rich layer around the stationary phase, which is known as a

pseudostationary phase, and an organic rich layer. As a result two modes of interaction are created; hydrophilic partitioning for retaining polar compounds, and weak electrostatic interactions between the charged analyte and the stationary phase, giving unique selectivity. The retention mechanism behind HILIC is not yet fully undertsood, but it is currently believed that retention is caused by partitioning [96]. Where the analyte differentially distibutes between the two layers, if it is more hydrophobic the anaylte will preferentially partition towards the mobile organic rich layer and be less retained and vice versa for a hydrophilic analyte (Figure 1.13 HILIC monolith interactions).

Figure 1.13 A HILIC mode scheme; HILIC interaction between analytes and monoliths in aqueous rich layer of the mobile phase



Monolithic columns are increasingly being used in separation science for the analysis of basic pharmaceutical drugs, because of their low resistance to mass transfer and high permeability as well as their easy preparation method and no

need for retaining frits [97]. Their stability at high pH is also an important feature. The high permeability of the monolithic stationary phases results from their high porosity, which in turn allows fast separations with high separation efficiencies [98]. There are many different types of HILIC monoliths ranging from polar/ neutral to cationic/ anionic monoliths. The most common type used for the separation of basic pharmaceuticals are zwitterionic monoliths, for example N, N-dimethyl-N-(2-methacryloyloxyethyl)-N-(3-sulphopropyl) ammonium betaine (SPE); the monomer and ethylene dimethacrylate (EDMA); the cross-linker, using azobisisobutyronitrile (AIBN) as free radical initiator to start the reaction between SPE and EDMA and the porogen methanol (MeOH) to create pores.

1.7 Research aims

HPLC methods for small molecules have been applied mainly with reversed-phase chromatographic chemistry and the current technology lacks the efficiency and specificity and is not environmental friendly analysis. It is the fact that an increase in column efficiency results from a reduction of column size. Our research will focus on using the capillary column and to achieve the better separation in nano-scale and may also improve column performance.

As discussed in the monoliths review, the structures of polymeric monolithic columns indicate their applications. They have been mainly studied on biomolecule separations with few on small molecule analysis. Organic monoliths contributed rapid and more capable separation than conventional packed HPLC columns (5 µm packed particles) for peptides analysis on a kinetic study by Guillarme et. al. [99]. They explained this was because of an improvement in mass transfer kinetics. However, the performance of organic monolithic columns was enhanced due to their small particle sizes.

Monoliths are typically produced by polymerisation of a cross-linking monomer and an active monomer, for example one containing functional groups with which the target analyte will react. It is important that the functional groups on the monolith impact the degree of binding with the target molecules. This will be investigated by the synthesis of a wide range of monoliths with different functional groups. There has been previously a fabricated wide range of monoliths in the KCL lab containing reactive epoxide groups which can then be used in turn to fabricate a wide variety

of analogues containing different functional groups including –OH, -COOH, -SO_3H, -$COOCH_2CH_2SO_3H$, -$NHCH_2NHCO$-, -$NHCH_2CHOHCH_2N^+R$ and –$COOCH_2CH_2N^+(CH_3)_3$- of particular interest when analysing small molecules are the HILIC materials and once again, the research group has considerable experience of fabricating these materials and these will be extensively evaluated for the project. These monoliths have the ability to offer many active bonding sites and together with different monomers will provide monoliths with wide ranging selectivities which is an obvious advantage for the methods. On the other hand, the physical characteristics of monolithic materials that can affect separation efficiency can easily be modified to suit the analysis of small molecules. With sample clean up not being as important when analysing small molecules in pharmaceutical industry, it is desirable to have materials with low flow resistance and where direct injection of samples can be achieved and efficient analysis is tailored by varying among other things, the type and composition of the porogens used. This is an area of research in which KCL lab has considerable experience due to extensive studies on monolith fabrication. The research will focus mainly on the use of acrylate and methacrylate compounds for monolith fabrication as they can easily be prepared and modified the active sites to suit the small molecule analysis.

The main aim in this research is to develop new monolithic stationary phases in a capillary column to cope with these drawbacks of the current applications [100], [101], [102], and [103]. The best monolithic column obtained from the project was

used to analyse small molecules (chorpheniramine maleate in commercial Pharmaceutical tablets) using micro-HPLC and CEC techniques.

Chapter 2

Experimental and method optimisation

Chapter 2. Experimental and method optimisation

Chapter 2 describes the validity of micro-HPLC and CE instruments using DiNa-Nano pump efficiency and electroosmotic flow (EOF) proving experiment, respectively. The screening and optimisation of the protocol for materials were also part of the validation. Core materials i.e., methacrylated HILIC monoliths, poly N, N-dimethyl-N-(2-methacryloyloxyethyl)-N-(3-sulphopropyl) ammonium betaine – co-ethylene glycol dimethacrylate (SPE-co-EDMA) were chosen as stationary phases for this preliminary phase. The optimisation and evaluation of the protocol include the fabrication of poly SPE-co-EDMA using micro-HPLC. Parameters such as polymerisation time, porogen percentage, initiators, thermal vs microwave polymerisation and length of column were optimised for the monolith.

The experiments in this chapter will focus on method optimisation for both micro-HPLC and CEC techniques. Due to using a DiNa Nano pump for micro-HPLC on a capillary column, a studying of the pump efficiency is essential. Factors affecting the organic monolith polymerisation were also studied and mobile phase optimisation for HILIC monolith was explained. For CE, the acids and bases separation were carried out to ensure the CE instrument works for the research.

2.1 Micro-HPLC experiments

The micro-HPLC with nano flow pump is the used to perform the following experiments for the research. It is very common to use for a study of monolithic column performance. Factors affecting the efficiency of solvent delivery (nano pump), the manufacture of poly organic HILIC (SPE-co-EDMA) monolith, comparison between a thermal and microwave polymerisations, the optimisation of mobile phase for HILIC separation, and a study of different initiator using for HILIC fabrication are described in the following experiments in this section.

2.1.1 Optimisation of DiNa nano flow pump using a flow meter

2.1.1.1 Introduction

In micro-HPLC, the reduction of the diameter of column from a classical size, 4.6 mm (i.d.) to a capillary size, 100 µm (i.d.) to get a better column performance is explained in Chapter 1. Thus a DiNa nano flow pump (Kya Tech) is used to deliver mobile phase for the experiment. This experiment was set up to ensure that the mobile phase pump was capable of delivering a low flow rate on the order of nL/min.

In practice, a conventional micro-HPLC pump can generate a nano flow by incorporating a post-pump flow-splitter to divert the excess flow. Alternatively, more modern ‘splitless’ systems may be used, which can provide accurate nano flow rates without the need for an additional splitter. There are thus two alternative methods to obtain nano flow rates:

a) split-system micro-HPLC and b) splitless micro-HPLC systems. Both of these are illustrated in Figure 2.1.

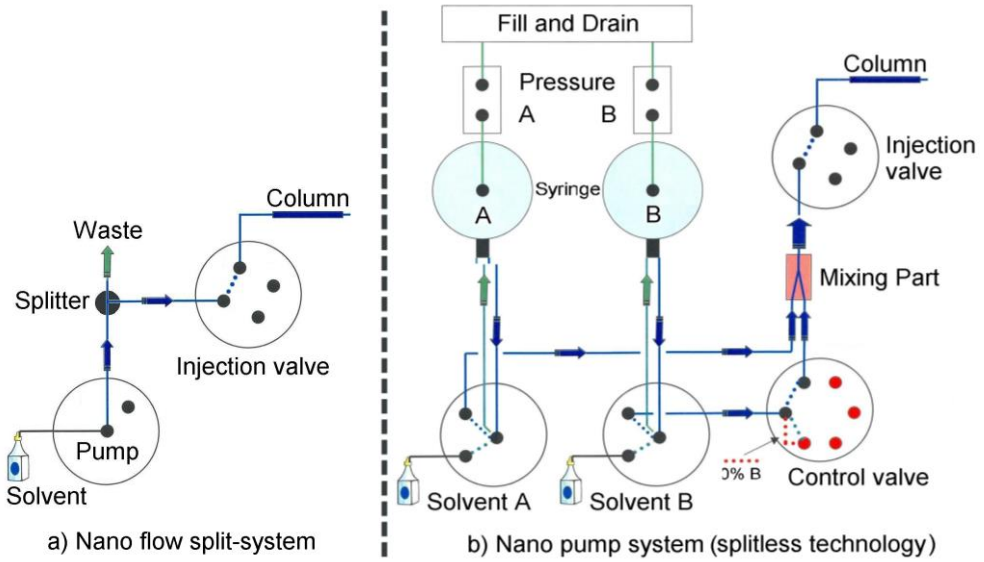


Figure 2.1 Nano flow pump systems. a) split-system micro-HPLC and b) nano flow pump system with splitless technology

This experiment was set to ensure micro-HPLC with DiNa nano pump is capable for small molecule analysis.

2.1.1.2 Experimental

2.1.1.2.1 Chemicals and materials

Ultrapure water (18.2 MΩ at 25 ºC) used for all pump performance tests was obtained from a Millipore Synergy® Ultrapure water system (Merck Millipore, MA, USA). A fused-silica capillary (20 µm x 375 µm x 25 cm) was purchased from Composite UK, Ltd. UK.

2.1.1.2.2 Instrumentation

A flow meter was made from an empty fused-silica capillary (20 µm x 375 µm x 25 cm) with micro-syringe, a micro-HPLC system with a DiNa non-split system (nano pump). Two types of a nano flow pump are described as follow;

a) Split-system micro-HPLC for nano flow

This system consists of an isocratic DiNa pump fitted with a four-port Valco injection valve and a 100-nL internal loop. The DiNa pump is a dual-piston reciprocating pump, with micro-syringe technology which controlled by a personal computer. A stainless steel T-piece with a flow-splitting capillary (50 µm (i.d.) x 30 cm length) was installed between the pump and the injection valve in order to divert the excess mobile phase. The actual flow rate of the system was determined by the split-rate ratio, which can be varied depending on the resistance of the waste outlet capillary and the resistance of the capillary column. Thus, the actual flow rate was determined by the pump flow rate and the split ratio, and was directly monitored from the outlet of the column.

b) Splitless nano flow pump system

This type of system consists of a DiNa nano gradient pump equipped with a four-port Valco injection valve with either a 100-nL internal loop or a 1 µL loop. The DiNa pumps were controlled separately using DiNa software (Clarity) [104]. On the DiNa system, solvents A and B are drawn and stored separately by syringe pump A and B respectively. To flush the pumps, fully filled syringes are used in the flushing mode, and solvents A and B are then drained out through the draining valve sets which are located next to the pressure regulators of each syringe. In

operation mode, solvent A delivers from the syringe pump directly to a mixing port (35 nL volume), whereas solvent B delivers through a control valve before being introduced to the same mixing port where both solvents mix and continue to the injection valve and column respectively.

2.1.1.2.3 Method

The measurement of DiNa nano pump efficiency experiment was carried out. To study the solvent delivery efficiency of the splitless nano flow DiNa pump, the DiNa pump was set to produce flow rates of 700, 1500, 3000, 4500 and 6000 nL/min with deionised (ultrapure) water as the mobile phase. A micro-syringe was attached to the outlet of the capillary and the accumulated output volume measured over a given period of time for each applied flow-rate. The output flow-rates were recorded along with the associated back pressures for all applied flow rates, and all measurements were performed in triplicate (i.e. flow rates and back pressures were measured six times for each applied flow rate).

2.1.1.3 Results and discussion

Table 2.1 lists the results for the splitless nano flow efficiency measurements, including measured volume consumption (nL), time consumption (minute), displayed back pressure (MPa) and measured flow rate (nL/min) when using different 5 fixed applied flow rates (nL/min).

Table 2.1 Flow meter experiment results on Dina Nano pump with DI water (n = 6)

Applied Flow Rate (nL/min)	Flow meter value at Time start (µL)	Flow meter value at Time stop (µL)	Volume consumption (µL)	Time consumption (minute)	Measured Flow Rate (nL/min)	Displayed Backpressure (MPa)
700	0.5	9.9	9400	13.51 ± 0.02	**695.78 ± 1.08**	**1.20 ± 0.01**
1500	0.4	7.8	7400	4.94 ± 0.02	**1497.98 ± 6.39**	**2.60 ± 0.01**
3000	0.9	9.6	8700	2.88 ± 0.02	**3020.83 ± 22.15**	**5.20 ± 0.01**
4500	0.1	9.9	9800	2.21 ± 0.01	**4434.39 ± 15.79**	**7.90 ± 0.01**
6000	0.8	9.4	8600	1.43 ± 0.01	**6013.99 ± 56.97**	**10.30 ± 0.01**

Results are an average of six separate readings (n = 6). To evaluate the efficiency of the splitless nano pump (DiNa nano pump) of micro-HPLC, the relationship of applied flow rates against the measured flow rate (Figure 2.2 A) and of applied flow rate against displayed backpressure (Figure 2.2 B) were determined.

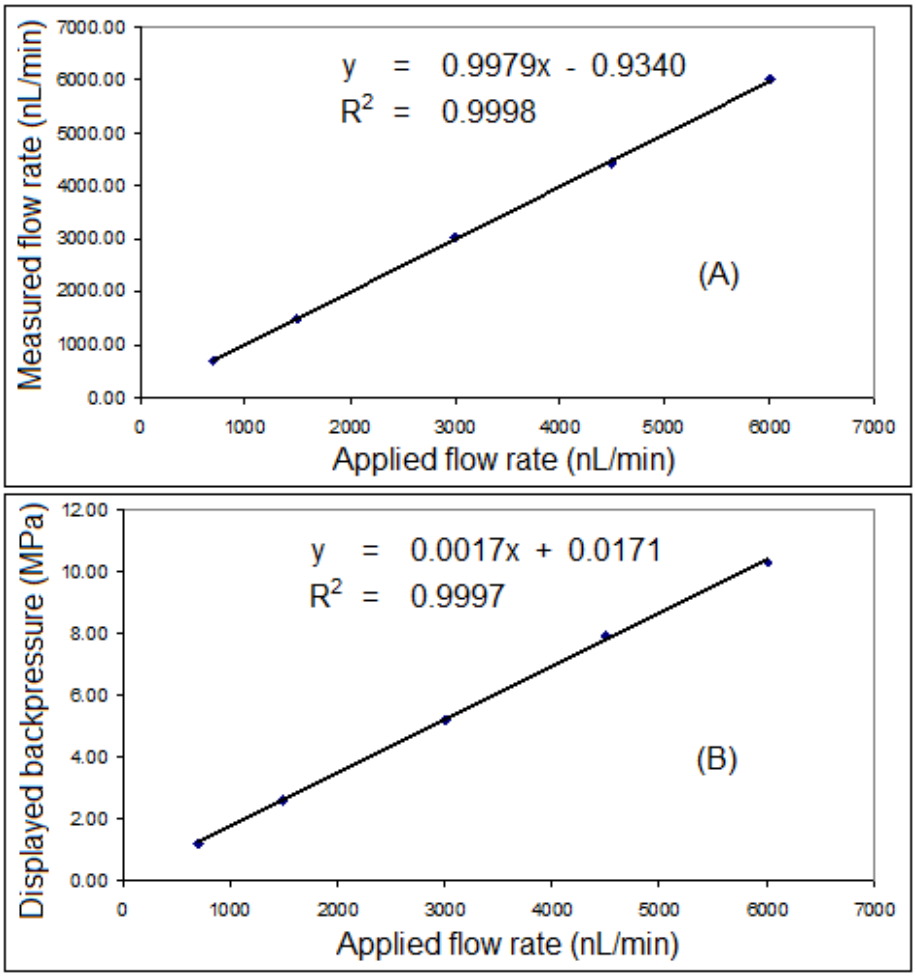


Figure 2.2 Relationship of (A) the applied flow rate (nL/min) against the measured flow rate (nL/min) and (B) the applied flow rate (nL/min) against the displayed backpressure (MPa) of a micro-HPLC DiNa nano pump (Splitless pump)

Firstly, the efficiency of the DiNa nano pump used in this work was tested. A flow meter was used to check if the measured flow rate being delivered by the pump in the micro-HPLC matched the applied flow rate. This was tested at flow rates between 700 nL/min and 6000 nL/min.

These results show that the measured flow rates of the DiNa nano pump are linearly related to the applied flow rates (A) with the high correlation coefficient (R^2 value) at 0.9998 and also mean displayed back pressures are directly relevant to fixed applied flow rates (B) with the high correlation coefficient (R^2 value) at 0.9997 (n = 6). These high correlation coefficients indicated the pump was delivering an actual flow rate that closely matched the applied flow rate. Therefore, the performance and repeatability of the DiNa pump delivery was reliable and acceptable to use for further micro-HPLC experiments.

2.1.2 Preparation of HILIC monolith using microwave and thermal polymerisation methods

2.1.2.1 Introduction

This experiment was established to compare between a microwave and thermal initiation polymerisation methods for preparation of HILIC monoliths. The experiment was set to study the effect of different polymerisation methods (microwave and thermal) on HILIC monolith efficiency. Both methods were generally used in many studies as in a literature review. The experiment will find out a better polymerisation method to suit the HILIC.

A monolithic column fabrication

There are five stages to preparing a monolithic column, *viz*. pre-treatment of the unmodified fused-silica capillary, preparation of the polymerisation mixture, filling the capillary with the polymerisation mixture, thermal initialisation or photochemical initialisation of polymerisation, and washing out un-reacted monomers and porogens [33]. These stages are generally depicted in the follow schematic illustration in Figure 2.3. The details of each step will be described after the Figure.

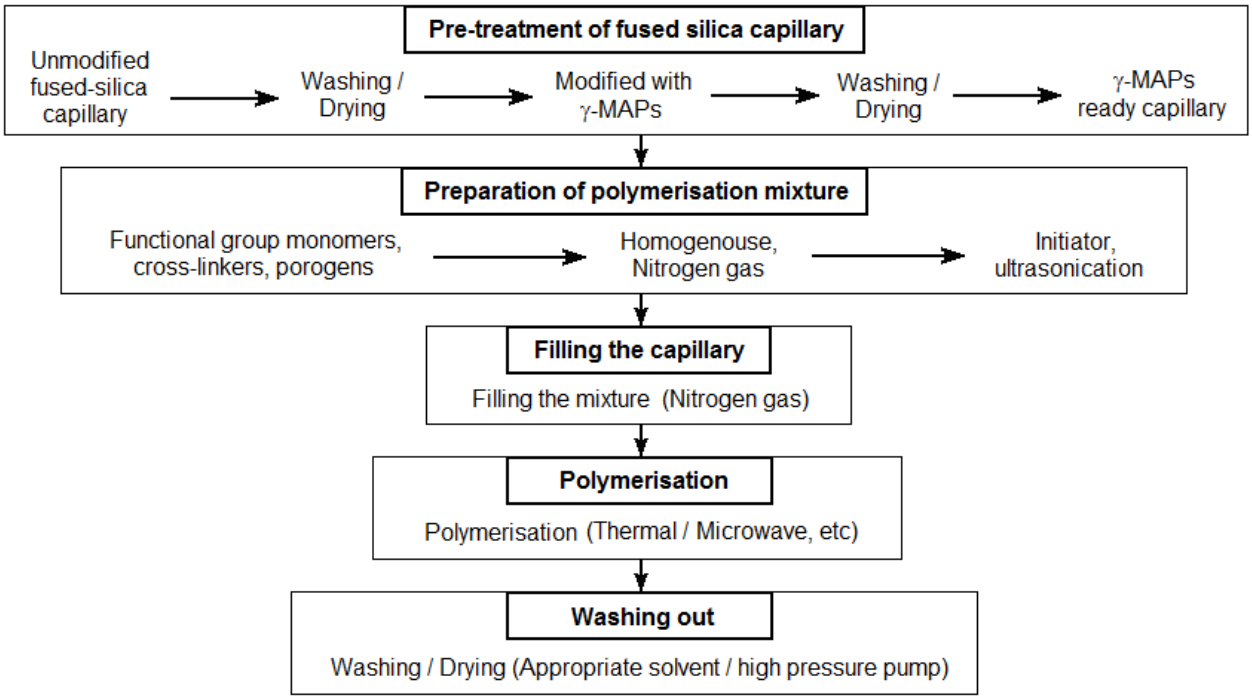


Figure 2.3 Steps in the fabrication of a typical monolithic column

2.1.2.1.1 Pre-treatment of fused silica capillary

Before use for monolith preparation, a new unmodified fused-silica capillary is pretreated to create anchoring sites on the inner wall of the capillary surface by adding 1 N sodium hydroxide then heating in the oven at 60 °C for 2 hours, washing with deionised water until getting neutral pH, and drying the column overnight by passing Nitrogen gas before the γ-MAPs polymerisation; these sites ensure that the monolith remains in the capillary when high pressure is applied. 3-(trimethyoxysilyl) propyl methacrylate (γ-MAPs) is generally used for this purpose. The methoxysilane of γ-MAPs reacts with the silanol groups on the inner fused silica surface, while the methacrylate group reacts with other substrates, permitting

functionalisation of the treated surface. Figure 2.4 depicts a typical silanisation reaction with γ-MAPs.

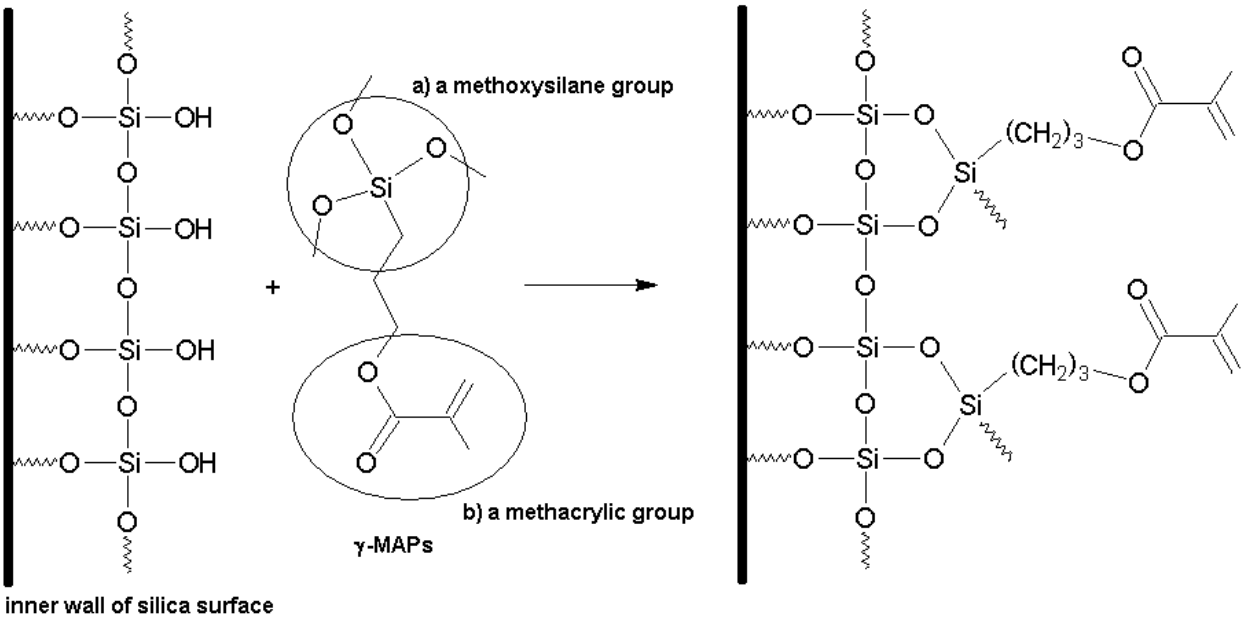


Figure 2.4 Silanisation reaction with γ-MAPs

2.1.2.1.2 Preparation of polymerisation mixture

The polymerisation mixture is composed of a functional monomer, a cross-linker monomer and a porogen, homogeneously mixed together using sonication. Generally, the monomers and porogen content will be measured in weight ratio or volume ratio. Nitrogen gas is passed through the mixture to degas it; this prevents unwanted inhibition of polymerisation in the presence of oxygen [33]. Once a homogeneous solution is obtained, the initiator (AIBN, 1% by weight of the monomers) is added into the solution, which is then ultrasonicated.

2.1.2.1.3 Filling the capillary

The homogeneous polymerisation mixture was introduced into the pre-treated capillary by applying nitrogen gas at a low pressure of around 20 to 60 psi to the vial container.

2.1.2.1.4 Polymerisation

The preparation of methacrylate-based monolithic columns is simple, and requires the use of polymerisation mixtures composed of a monomer, a cross-linker, a porogen and the polymerisation initiator. The monomers used are methacrylate esters such as glycidyl methacrylate (GMA) and 2-hydroxyethyl methacrylate, and ethylene glycol dimethacrylate (EDMA) is added as a cross-linker that offers a derivatisable part of the monolith. Porogens are commonly a mixture of alcohols, such as methanol and propanol. AIBN is a desirable initiator and can be activated by either a thermal method or a UV light process [11]. The mixture is then introduced into the pre-treated fused silica capillary and placed in an oven at an elevated temperature, whereby the macro-pores, cross-linking and rigid monolithic polymers are formed. As shown in Figure 2.5, the mechanism of the polymerisation reaction can be described in two steps: a) growth of the polymer chain and b) cross-linking by EDMA.

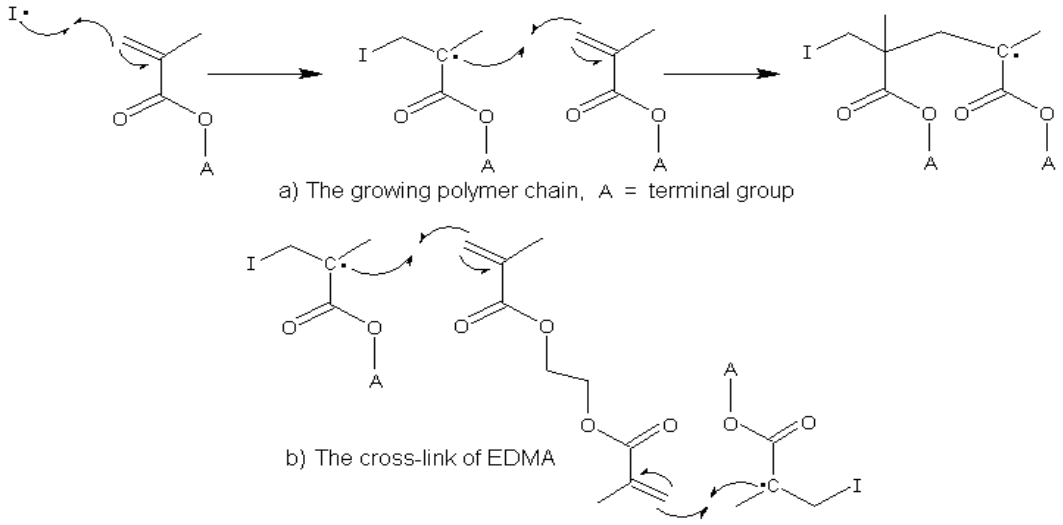


Figure 2.5 Mechanisms of methacrylate-based monolithic polymerisation reaction

2.1.2.1.5 Washing out

After completion of polymerisation, un-reacted components should be washed out. The monolithic column is usually washed with methanol using a HPLC pump operating at 1000- 6000 psi, in order to remove both porogenic solvent and other un-reacted soluble compounds.

2.1.2.2 Experimental

2.1.2.2.1 Chemicals and materials

3-(trimethyoxysilyl) propyl methacrylate (γ-MAPS), N,N-dimethyl-N-(2-methacryloyloxyethyl)-N-(3-sulphopropyl) ammonium betaine (SPE), ethylene glycol dimethacrylate (EDMA), 2, 2'-azoisobutyronitrile (AIBN), ammonium hydroxide, acrylamide, thiourea and toluene were purchased from Sigma-Aldrich (Dorset, UK). HPLC-grade methanol and acetonitrile were obtained from Fisher Scientific (Leicestershire, UK). Deionised water was produced using a Millipore System (part no. SYNSV000). Ammonium formate was purchased from VWR

International; Leicestershire, UK. Fused silica capillary with 100 µm (i.d.) x 375 µm (o.d.) was purchased from Composite Metal Services (Shipley, UK).

2.1.2.2.2 Instrumentation

A Genlab oven was used for thermal polymerisation (Cheshire, UK) and a Hinari microwave oven with 700 W output power was used for microwave polymerisation. An Applied Biosystems (ABI 400) was used to flush capillaries. The experiment was carried out with a custom-built HPLC system, consisting of a 'DiNa S' isocratic pump (DiNa Isocratic, KYA Tech Corporation), a four-port injection valve with a 100-nL internal loop (Valco) and an Applied Biosystems 757 absorbance detector with a detection wavelength of 214 nm. The actual flow rate was determined from the volume of eluent collected during a certain period of time (as described in Experiment 2.1.1). A Clarity chromatography data station (DataApex) was used for data acquisition and data handling. Chromatograms were converted to ASCII files and redrawn using Microsoft Excel.

2.1.2.2.3 Method

Six unmodified fused silica capillaries were pre-treated with γ-MAPS, and then used to prepare HILIC monoliths using a polymerisation mixture containing SPE and EDMA, with methanol as the porogen solvent and AIBN as the initiator. The proportion of monomers and porogen solvent used are shown in Table 2.2, while the polymerisation reaction for the HILIC, poly (SPE-co-EDMA) monolithic column is displayed in Figure 2.6.

Table 2.2 Composition of polymerisation mixture used for preparation of poly (SPE-co-EDMA) monolithic column

Monomer mixture (%, w/w)		Porogen solvent (%, w/w)	Polymerisation mixture (%, w/w)		
SPE	EDMA cross-linker	MeOH	Monomer mixture	Porogen solvent	Initiator, AIBN
43.0	57.0	100.0	33.25	66.75	1.00

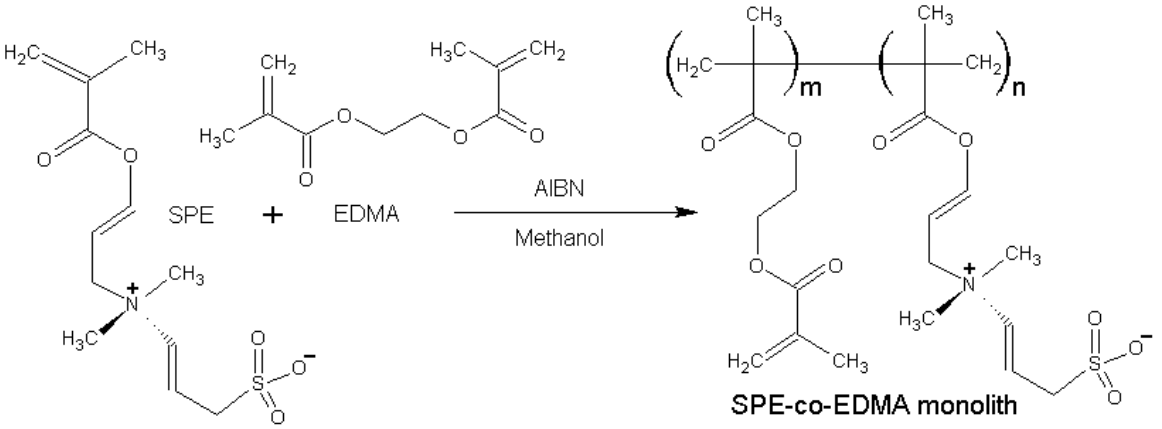
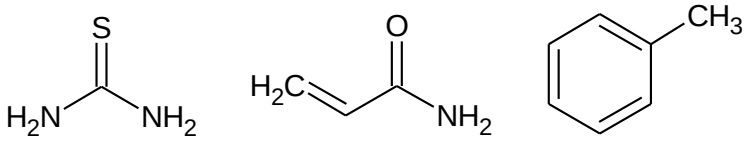


Figure 2.6 Polymerisation reaction for poly (SPE-co-EDMA) monolithic column

The two sets of columns used for comparison of initiation methods were designated A and B; each contained 3 pre-treated columns. Columns in set A were polymerised using microwave irradiation, set at 750 W power for 25 minutes, and columns in set B were polymerised at 60 °C in a Genlab oven for 12 hours. After the polymerisation was completed, both sets of monoliths were washed out with methanol, deionised water and dried overnight by flushing with dry nitrogen gas. Both sets of capillaries were cut to a total length of 35 cm with the desired effective length about 30 cm, then 2-3 mm detection window was created at a distance of 2-3 cm from the end of the column using a thermal wire stripper. The monolithic

material was then pyrolysed and flushed out with methanol. The prepared columns each had dimensions 30 cm x 100 µm (i.d.) x 375 µm (o.d.).

In order to compare the effect of initiation method on chromatographic performance, each HILIC monolith was used to separate a test mixture varying from non-polar, neutral and polar compounds containing toluene, acrylamide and thiourea respectively (Fig. 2.7). The mobile phase used was acetonitrile: (5 mM ammonium formate, pH 3.0) combined in a 9:1 ratio. To prepare the mobile phase, a stock solution of 5 mM ammonium formate was adjusted to the desired pH value of 3.0 with 5 M formic acid. Stock solutions of test compounds were made in ACN to obtain a final concentration of 1 mg/mL; these were then diluted to the appropriate concentration of 100-200 µg/mL in the test mixture. The test mixture was analysed on both sets of columns using a flow rate of 1000 nL/min, an injection volume of 100 nL, and peaks were detected using UV-vis at 214 nm. The numbers of the theoretical plates (*N*) for each peak were calculated to compare the efficiency of monoliths prepared by the two different polymerisation methods.



Thiourea Acrylamide Toluene

Figure 2.7 Chemical structures of thiourea, acrylamide and toluene

2.1.2.3 Results and discussion

Table 2.3 lists the retention times and calculated the numbers of the theoretical plates (*N*) for each test compound (toluene, acrylamide and thiourea) on each column from sets A and B. Example chromatograms obtained from each set are shown in Figure 2.8.

Table 2.3 Retention times and the numbers of the theoretical plates (*N*) for test compounds on poly (SPE-co-EDMA) monolithic columns prepared via (A) microwave initiation method and (B) thermal initiation method

Column	Column length (cm)	Pressure (MPa)	Toluene		Acrylamide		Thiourea	
			t_R(min)	*N* (per metre)	t_R(min)	*N* (per metre)	t_R(min)	*N* (per metre)
Microwave polymerisation								
A1	30.6	11.5	3.20	4507.1 ± 20.4	3.79	4199.7 ± 21.8	8.43	5819.4 ± 62.9
A2	30.2	10.9	3.16	4431.7 ± 25.1	3.75	4177.8 ± 27.2	8.40	5796.5 ± 48.9
A3	30.5	10.7	3.18	4490.2 ± 14.3	3.76	4169.4 ± 17.3	8.44	5827.9 ± 71.3
Mean ± SD				**4476.3 ± 19.9**		**4182.3 ± 22.1**		**5814.6 ± 61.0**
%RSD				**0.85**		**0.81**		**0.90**
Thermal polymerisation								
B1	29.7	6.9	3.02	5510.2 ± 22.9	3.52	5419.4 ± 26.7	7.33	7789.9 ± 58.6
B2	29.5	6.7	3.00	5542.8 ± 24.9	3.49	5504.6 ± 20.4	7.29	7801.3 ± 67.3
B3	30.2	7.2	3.05	5552.2 ± 17.4	3.55	5612.4 ± 31.1	7.37	7868.1 ± 57.8
Mean ± SD				**5535.1 ± 21.7**		**5512.1 ± 26.1**		**7819.8 ± 61.2**
%RSD				**0.86**		**0.20**		**0.67**

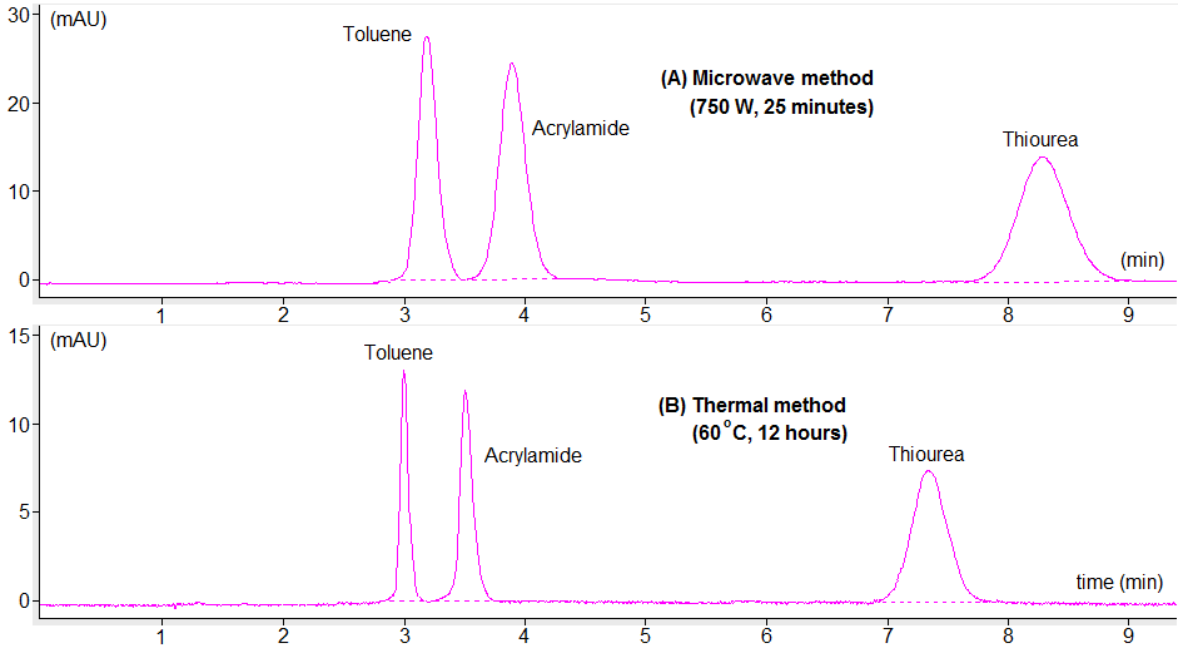


Figure 2.8 Chromatograms for test mixture separation using poly (SPE-co-EDMA) columns prepared via (A) microwave method versus (B) thermal method

The HILIC behavior of the three test compounds were used to determine chromatographic performance of the prepared monoliths. Thiourea, which is normally used as a dead volume marker in RP-HPLC, eluted after toluene and acrylamide on the HILIC monoliths. This can be explained due to the polarity differences between the three compounds: toluene as being a nonpolar compound eluted first, acrylamide as a neutral compound eluted thereafter and thiourea as a polar compound was eluted last (Figure 2.8).

Moreover, the results from Table 2.3 showed significant differences for all three compounds in both retention times (t_R) and N values on monoliths prepared by the microwave method (A) or the thermal method (B). Mean retention times of toluene, acrylamide and thiourea were 3.18, 3.77 and 8.46 minutes for the microwave

method and 3.02, 3.52, and 7.33 minutes for the thermal method. Mean the numbers of the theoretical plates (*N*) of toluene, acrylamide and thiourea peaks were 4476.3, 4182.3 and 5814.6 plates per meter for microwave method and 5535.1, 5512.1, and 7819.8 plates per meter for thermal method, with standard deviations (SD) and percentage of relative standard deviations (%RSD) showed in Table 2.2 respectively. Based on these values for *N*, it is clear that the thermal initiation method generates a more efficient monolithic column than the microwave method.

2.1.3 Optimisation of mobile phase for the monolithic HILIC separation using thermal polymerisation method

2.1.3.1 Introduction

To study the HILIC retention mechanism using three different proportions of a high organic content mobile phase, this experiment was set to find the suitable mobile phase for the monolithic HILIC column to separate HILIC test compounds giving best column performances.

2.1.3.2 Experimental

2.1.3.2.1 Chemicals and materials

All chemicals and materials used were as listed in section 2.1.2.2.1

2.1.3.2.2 Instrumentation

Instrumentation used was as described in section 2.1.2.2.2

2.1.3.2.3 Method

Three sets of unmodified fused silica capillaries were derivatised by γ-MAPS and used for preparation of HILIC monoliths based on the SPE and EDMA mixture, with methanol as a porogenic solvent and AIBN as an initiator. The proportion of monomers and porogen used were the same as in the previous experiment (Table 2.2). The thermal method was used for initiation of polymerisation, as it obviously produced better column efficiency than the microwave method (section 2.1.2.2.3).

The mixtures were polymerised in the pre-treated columns at 60 ºC in a Genlab oven for 12 hours (n = 3). After completion of polymerisation with a detection window, the same dimension of column was prepared and the test mixture used in section 2.1.2 were analysed using the chromatographic conditions described

before (section 2.1.2.4). All stock solutions and test compound solutions were prepared as in the previous experiment. Three different mobile phase compositions were prepared, consisting of acetonitrile combined with 5 mM ammonium formate (pH 3.0) in three different ratios (v/v): 40: 60, 50: 50 and 90: 10 respectively. Finally, chromatograms from three different mobile phase compositions were then compared in terms of the HILIC retention mechanism.

2.1.3.3 Results and discussion

Results of the experiment including the numbers of the theoretical plates (*N*) and retention times for three test compounds (toluene, acrylamide and thiourea) at three different ratios of acetonitrile to buffer are presented in Table 2.4. Each value was calculated as an average of three separate measurements.

Table 2.4 Retention times and the numbers of the theoretical plates (*N*) for test compounds on poly (SPE-co-EDMA) monolithic columns, using three different ratios of acetonitrile to ammonium formate buffer

Column	Mobile phase Proportion	Pressure (MPa)	Toluene	Acrylamide	Thiourea
			N (per metre)/ [t_R (min)]	*N* (per metre)/ [t_R (min)]	*N* (per metre)/ [t_R (min)]
1	40 / 60	12.4	5216.2 ± 22.3 [3.71 ± 0.02]	4986.3 ± 34.1 [4.05 ± 0.04]	6814.8 ± 44.8 [5.75 ± 0.04]
2	50 / 50	9.8	5096.7 ± 20.1 [3.65 ± 0.02]	5199.6 ± 21.9 [3.89 ± 0.04]	7419.5 ± 66.7 [5.90 ± 0.05]
3	90 / 10	6.9	5546.1 ± 31.0 [3.02 ± 0.01]	5518.5 ± 29.2 [3.52 ± 0.03]	7823.7 ± 54.9 [7.34 ± 0.07]

Generally, HILIC separations employ water and acetonitrile in the mobile phase, but require much higher organic content (>60%) in order to ensure significant

hydrophilic interaction [30]. In this experiment, the percentage of ACN was varied between 40-90%, while the ammonium formate pH 3.0 was set at 5 mM (due to the lower salt solubility in mobile phases containing high acetonitrile content). The influence of the acetonitrile content in the mobile phase on the retention time of three test compounds is shown in Figure 2.9. As evident from Table 2.4, retention on the HILIC monoliths increases with increasing ACN content.

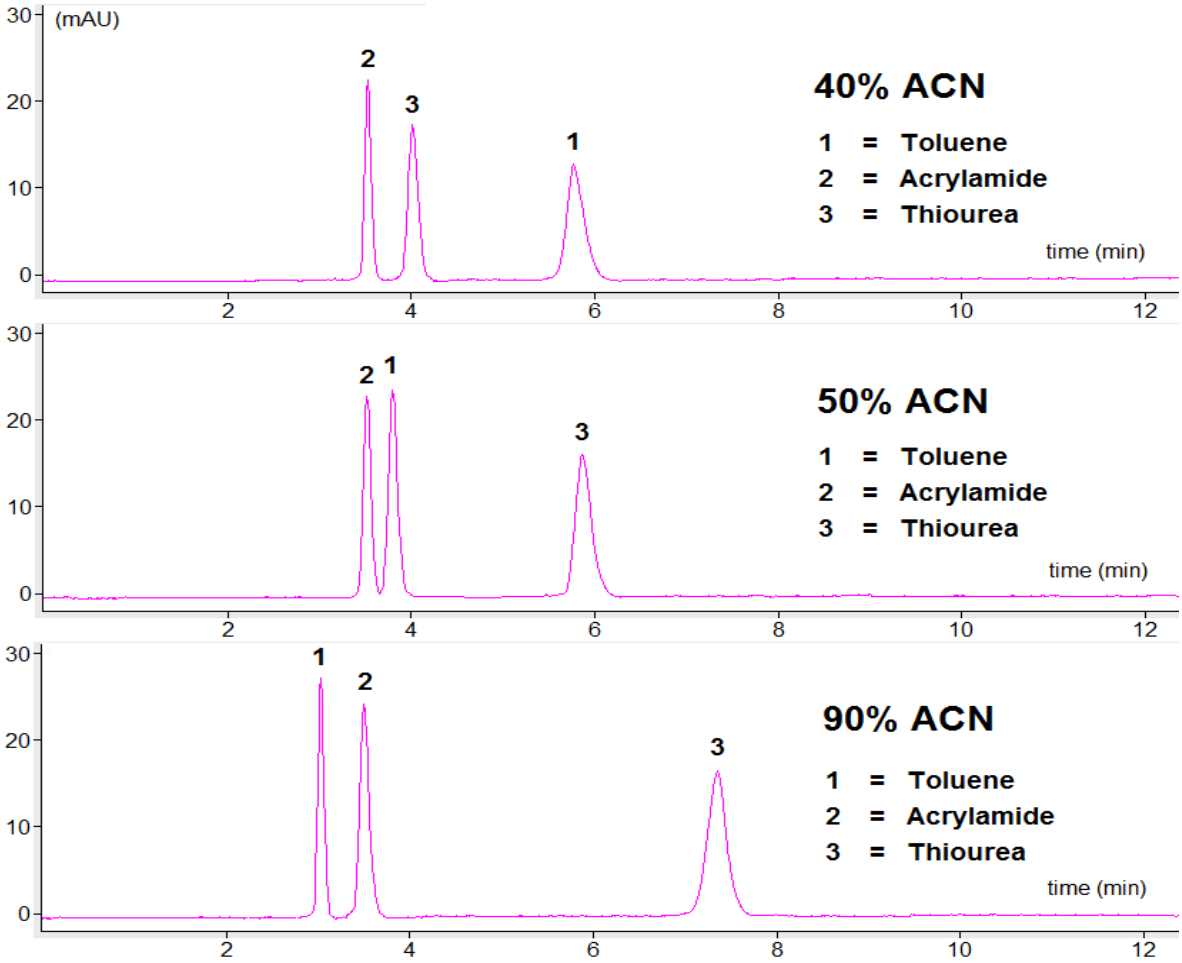


Figure 2.9 Separation of test mixture on poly (SPE-co-EDMA) monolithic column using three different proportions of acetonitrile in the mobile phase

As shown in Figure 2.9, thiourea (the most polar compound in the test mixture) eluted after toluene and acrylamide when ACN content was increased from 40% to

90%. The retention time of thiourea decreased when ACN content in the mobile phase decreased from 90% to 50% v/v, and then remained relatively constant as ACN content was decreased further to 40%. For the non-polar compound toluene, which was eluted first at high ACN content, significantly different results from thiourea were observed. At lower percentages of ACN (<50%), toluene retention times increased dramatically. Acrylamide, a neutral compound, behaved similar to thiourea but with smaller retention time shifts. These results demonstrate a typical HILIC retention mechanism only at the higher ACN content. It is quite interesting that at lower percentages of acetonitrile, the order of elution suggests a reverse phase rather than a HILIC mechanism.

This complicates prediction of elution order for these monoliths, and might even call into question the “HILIC behavior” of the monolith. This “double behavior property” could be exploited further in to expand the range of applicability of this monolith, as it appears that not only pH but also the percentage of organic solvent in the mobile phase can change the order of elution of small compounds. This characteristic will be further explored in Chapter 6 concerning method development for basic small molecule experiments.

Another observation to note is the high efficiency of the neutral compound, this efficiency is not changed with retention time and it is very high. This was a reproducible phenomenon, which is very difficult to explain theoretically. This can be predicted that the possible charges and polarisability of both phase and compounds needs fine-tuning and that the system is very sensitive to small solvent changes.

2.1.4 Effect of different initiators (AIBN and V-501) on column efficiency

2.1.4.1 Introduction

Most of researchers mainly reported AIBN used as an initiator for preparation of organic monolithic polymerisation. In our lab, we also have AIBN and V-501 to use for the polymerisation reaction. In this experiment we aim to study the effect of different initiators (AIBN and V-501) on a monolithic HILIC (poly SPE-co-EDMA) column efficiency.

2.1.4.2 Experimental

2.1.4.2.1 Chemicals and materials

Chemicals and materials used were as listed in section 2.1.3.2.1, with the addition of 4, 4'-azobis (4-cyanopentanoic acid) (V-501) as another initiator was purchased from Sigma-Aldrich, USA.

2.1.4.2.2 Instrumentation

Instrumentation used was as described in section 2.1.3.2.2

2.1.4.2.3 Method

The same procedure of HILIC monolithic preparation as Experiment 2.3 was followed. Six unmodified fused silica capillaries were pre-treated with γ-MAPs, then filled with polymerisation mixture containing SPE and EDMA, with methanol as a porogenic solvent. Two different initiators were used: AIBN and V-501, at 1% w/w (both AIBN and V-501) and 1.7% (V-501 only). Thermal polymerisation was performed for 12 and 24 hours in each case. The proportion of monomers, the porogen solvent used and the analysis conditions were the same as in Table 2.2. After finishing the HILIC columns preparation, columns were evaluated with a

same standard test mixture of toluene, acrylamide and thiourea under the same chromatographic conditions as section 2.1.2.4 (mobile phase; ACN: 5 mM ammonium formate pH 3.0 (90: 10), 214 nm, flow rate 1000 nL/min). Finally, chromatograms from six columns were calculated in term of the numbers of the theoretical plates (*N*) and compared the efficiency of two initiators using in the thermal polymerisation. All measurements were performed in triplicate.

2.1.4.3 Results and discussion

Experimental results including retention times and the numbers of the theoretical plates (*N*) for three test compounds (toluene, acrylamide and thiourea) on columns prepared with two different initiators (AIBN and V-501) are presented in Table 2.5. Figure 2.10 shows the analysis of the three compound test mixture under all six different polymerisation conditions studied here.

Table 2.5 Retention times and the numbers of the theoretical plates (*N*) for test compounds on poly (SPE-co-EDMA) monolithic columns prepared using two different initiators (1% AIBN, 1% V-501 and 1.7% V-501 in mobile phase; ACN: 5 mM ammonium formate pH 3.0 (90: 10)

Column	Polymerisation time (hour)	Initiator	%	Column length (cm)	Pressure (MPa)	Toluene	Acrylamide	Thiourea
						N/m (Mean ± SD) [t_R (min)]	*N/m (Mean ± SD)* [t_R (min)]	*N/m (Mean ± SD)* [t_R (min)]
1	12	AIBN	1.0	31.5	6.9	5528.3 ± 8.9 [3.03 ± 0.01]	5597.8 ± 10.4 [3.52 ± 0.02]	7819.5 ± 19.8 [7.33 ± 0.06]
2	24	AIBN	1.0	31.0	10.0	4421.1 ± 7.9 [2.70 ± 0.01]	5281.3 ± 8.6 [3.18 ± 0.01]	6317.2 ± 20.8 [6.46 ± 0.05]
3	12	V-501	1.0	31.0	6.8	4254.4 ± 8.4 [2.55 ± 0.01]	4975.9 ± 9.7 [3.16 ± 0.01]	6214.5 ± 17.1 [6.48 ± 0.07]
4	24	V-501	1.0	28.0	11.0	4120.9 ± 6.2 [2.40 ± 0.01]	4641.4 ± 7.1 [3.03 ± 0.02]	6118.4 ± 24.6 [6.10 ± 0.05]
5	12	V-501	1.7	29.6	4.4	4902.8 ± 10.2 [2.49 ± 0.01]	4989.7 ± 17.7 [3.12 ± 0.02]	6477.1 ± 25.4 [6.83 ± 0.06]
6	24	V-501	1.7	31.3	6.6	4619.1 ± 12.6 [2.73 ± 0.01]	4667.7 ± 16.0 [3.28 ± 0.02]	6240.9 ± 31.6 [6.85 ± 0.05]

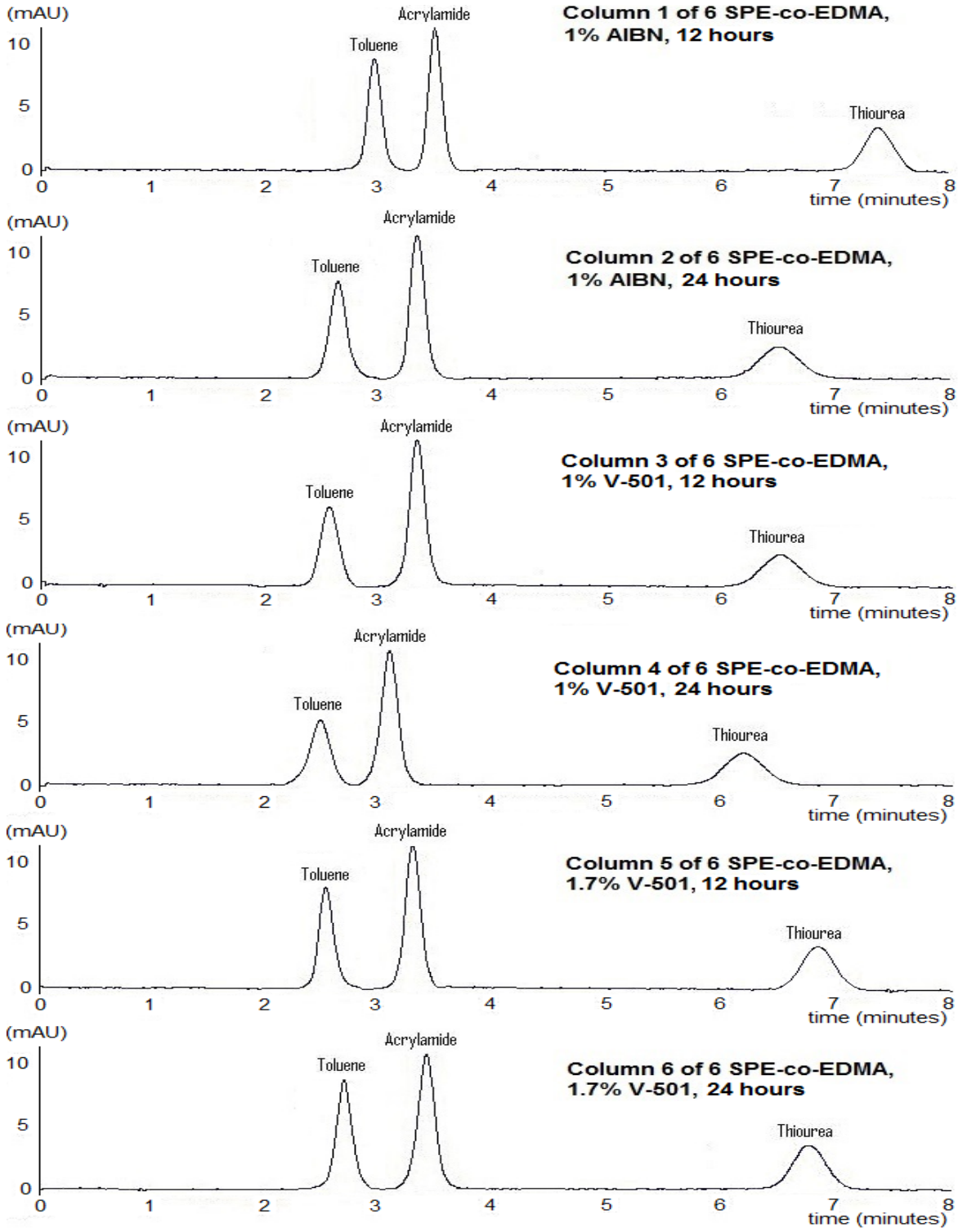


Figure 2.10 Separation of test mixture on poly (SPE-co-EDMA) monolithic columns, prepared with varying types and amounts of initiators for different polymerisation times

As shown in Table 2.5, significant differences in the average retention times (t_R) and the numbers of the theoretical plates per metre (N/m) of the three test compounds were observed on columns prepared using different initiators. The best overall performance was obtained when polymerisation was performed for 12 hours using 1% AIBN.

However, there were some relevant differences for displayed backpressures, means of the numbers of the numbers of the theoretical plates (N) and with time of polymerisation. When polymerisation was performed for 24 hours, columns prepared with both AIBN and V-501 initiators showed higher backpressures, which may be attributable to porosity differences between them. Longer polymerisation times lead to monoliths with lower porosities, which in turn results in higher backpressures.

It was concluded that polymerisation for 12 hours was optimal, since the average the numbers of the theoretical plates (N) for columns prepared by this method was significantly higher than that of the columns prepared by polymerisation for 24 hours, regardless of initiator used. Furthermore, columns prepared with AIBN had higher average N values when compared to those prepared using V-501 as initiator. Based on these results, the optimal conditions for the preparation of the poly (SPE-co-EDMA) monolithic column include using 1% AIBN as an initiator and thermal polymerisation for 12 hours. Furthermore, characterisation of monoliths with Scanning Electron Microscopy (SEM) is commonly used as a tool to evaluate porosity and characterise the prepared monoliths. It may be performed if would have been interesting to see the differences in morphology of monoliths.

2.2 CE experiments

As mentioned CE principle in Chapter 1, CE is an instrument for small molecules analysis with better column performance than micro-HPLC's because of the plug-like profile of EOF driving forces. In CE separation, analytes will move along the column because of their charges in a background electrolyte (BGE) and their masses or molecular weights. The mobility of analytes can be named as apparent mobility whereas the real mobility of it is called effective mobility. This effective mobility can be calculated from apparent mobility subtracting with the EOF mobility from the BGE. These factors are important to calculate the mobility of background electrolyte and velocity of analytes in the capillary column.

From the CE principle, there are three equations below to use for calculation of effective mobility (μ_{eff}), measured mobility (μ_{app}), EOF mobility (μ_{EOF}) to study the CE separation of analyte.

$$\mu_{eff} = \mu_{app} - \mu_{EOF} \quad \text{Equation 13}$$

$$\mu_{eff} = (L_d * L_t) / (t_{m(i)} * V) \quad \text{Equation 14}$$

$$\mu_{app(EOF)} = (L_d * L_t) / (t_{m(EOF)} * V) \quad \text{Equation 15}$$

where as μ_{eff}, is an effective mobility, μ_{app} is a measured mobility, μ_{EOF} is an EOF mobility, L_t is a total length of capillary, L_d is an effective length of capillary, t_m is a migration time and V is an applied voltage [95].

In these CE experiments, acid and base CE separations were performed to demonstrate the use of CE principle including those three equations (Equation 13 – 15). After the CE separation, the utility of equations was exhibited with the mobility values of compounds which are related to the orderity of separation.

2.2.1 EOF study of acidic compounds and their CE separation using normal polarity at basic pH, 8.0

2.2.1.1 Introduction

The experiment was set to study EOF of background electrolyte and to analyse three acids (benzoic acid and two benzoic acid derivatives) employing CE principle at a high pH condition in normal polarity mode of CE. This experiment also was carried out to ensure CE technique will fulfill analysis of small molecules for the research.

2.2.1.2 Experimental

2.2.1.2.1 Chemicals and materials

Benzoic acid, salicylic acid, 3, 4, 5-trihydroxybenzoic acid, thiourea, sodium hydroxide, and boric acid were all purchased from Sigma-Aldrich (Dorset, UK). Fused silica capillary with a dimension of 100 µm (i.d.) x 375 µm (o.d) was obtained from Composite Metal Services (Shipley, UK).

2.2.1.2.2 Instrumentation

All measurements were performed on a Hewlett-Packard 3D-CE, UK with Chemstation software for data acquisition and data handling. Resultant electropherograms were converted to an ASCII file and redrawn using Microsoft Excel.

2.2.1.2.3 Method

CE analysis of acidic compounds was carried out using normal polarity mode and a fused silica capillary with the following dimensions: a total length of 45 cm, an effective length of 36.5 cm, and 100 µm (i.d.) x 375 µm (o.d.). The background

electrolyte (BGE) was 50 mM sodium borate pH 8.0, the temperature was set at 25 °C, and peaks were detected by UV at 210 nm. Injection was carried out by applying a pressure of 10 mBar for 5 seconds and an applied potential of +30.0 kV using normal polarity CE. Electroosmotic flow mobility (EOF) was determined by injection of thiourea at 30 µg/mL. EOF value, effective mobility, and measured mobility of each compound were calculated using equations 13, 14 and 15. The test solution was prepared at a final concentration of 100 µg/mL in the BGE. Figure 2.11 shows the chemical structures of the test compounds, *viz.* benzoic acid, salicylic acid, and 3, 4, 5-trihydroxybenzoic acid.

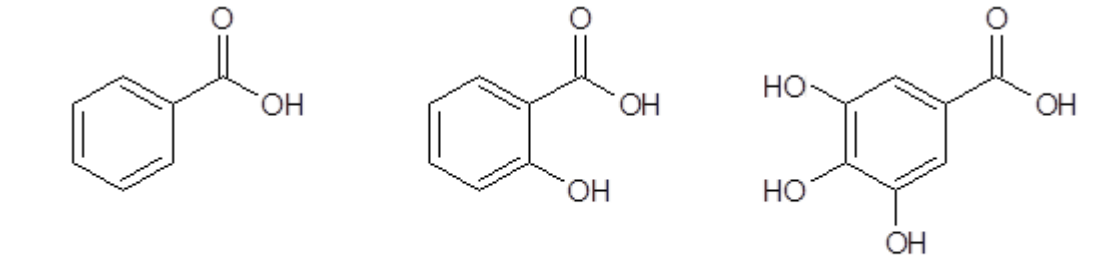


Figure 2.11 Chemical structures of acidic test compounds benzoic acid, salicylic acid and 3, 4, 5-trihydroxybenzoic acid for normal polarity CE measurements

2.2.1.3 Results and discussion

This electropherogram in Figure 2.12 shows the EOF marker thiourea, as it appears using normal polarity CE in BGE buffer at pH 8.0.

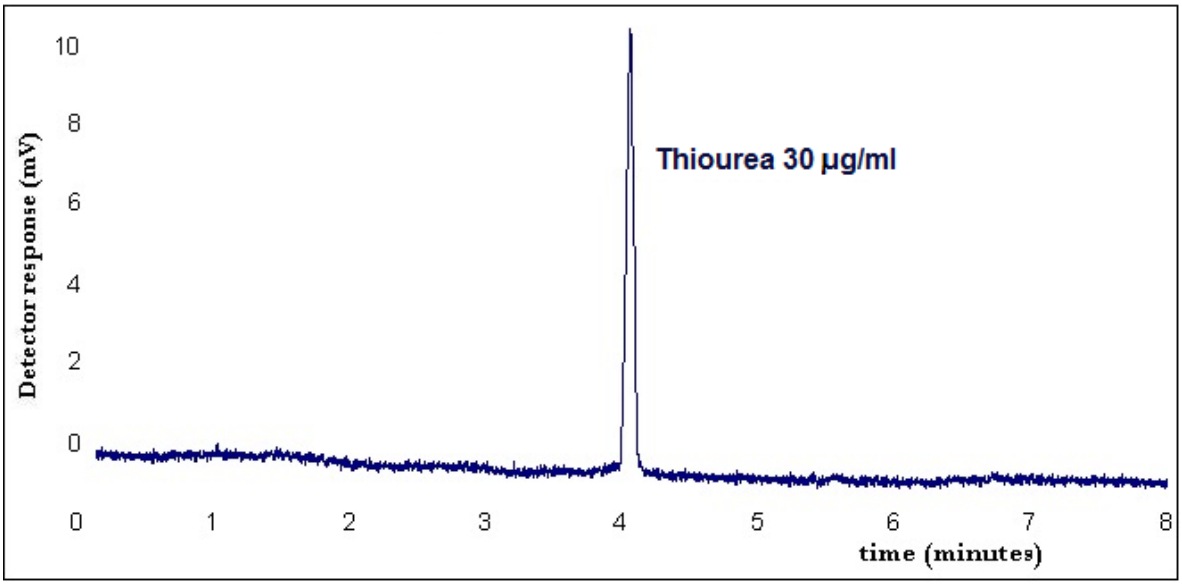


Figure 2.12 Electropherogram of thiourea (EOF marker) using normal polarity CE, with 50 mM sodium borate at pH 8.0 as BGE, +30 kV applied voltage

For normal polarity CE using a BGE of 50 mM sodium borate (pH 8.0) and +30.0 kV applied voltage, acidic compounds including benzoic acid, salicylic acid, and 3, 4, 5-trihydroxybenzoic acid were fully negatively charged, therefore their electrophoretic mobility was towards the anode. However, the strong EOF resulted in these analytes being detected at the cathode. Their separation comes as a result of differences in their pKa values, producing differing degrees of ionisation and hence differing mobilities. The electroosmotic flow mobility (μ_{EOF}), effective mobility (μ_{eff}), and measured mobility (μ_{app}) are displayed in Table 2.6.

Table 2.6 Electrophoretic mobilities (x 10^{-4} cm^2 $V^{-1}s^{-1}$, absolute value) and migration times of benzoic acid, salicylic acid, and 3, 4, 5-trihydroxybenzoic acid at pH 8.0

Electropherogram number	EOF	Benzoic acid			Salicylic acid			3 ,4, 5-trihydroxybenzoic acid		
	μ_{EOF}	t_m (min)	μ_{app}	μ_{eff}	t_m (min)	μ_{app}	μ_{eff}	t_m (min)	μ_{app}	μ_{eff}
1	2.231	4.68	1.950	0.281	5.56	1.641	0.590	6.88	1.326	0.905
2	2.220	4.70	1.941	0.279	5.61	1.627	0.593	6.91	1.321	0.899
3	2.224	4.74	1.925	0.299	5.64	1.618	0.606	6.97	1.309	0.915
4	2.219	4.69	1.946	0.273	5.57	1.638	0.581	6.94	1.315	0.904
5	2.228	4.72	1.933	0.295	5.63	1.621	0.607	6.99	1.305	0.923
6	2.226	4.70	1.941	0.285	5.62	1.624	0.602	6.93	1.317	0.909
MEAN	**2.225**	**4.71**	**1.939**	**0.285**	**5.61**	**1.628**	**0.597**	**6.94**	**1.316**	**0.909**
SD	**0.0046**	**0.0217**	**0.0089**	**0.0098**	**0.0327**	**0.0095**	**0.0104**	**0.0398**	**0.0076**	**0.0084**
%RSD	**0.21**	**0.46**	**0.46**	**3.43**	**0.58**	**0.59**	**1.75**	**0.57**	**0.57**	**0.92**

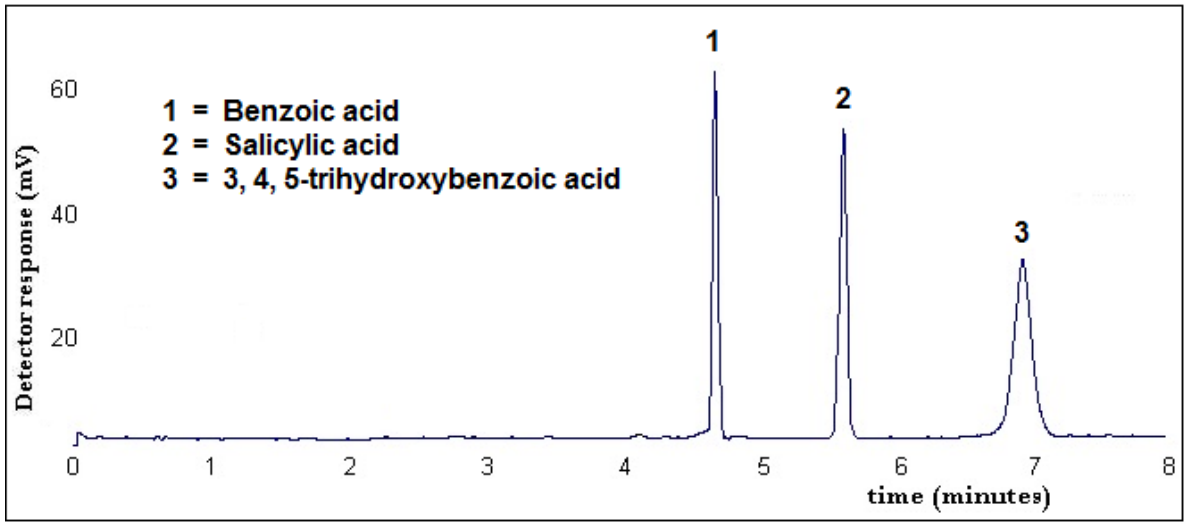


Figure 2.13 Electropherogram of benzoic acid, salicylic acid and 3, 4, 5-trihydroxybenzoic acid (acidic compounds) using normal polarity CE, with 50 mM sodium borate (pH 8.0) as BGE, +30 kV applied voltage

Under these high pH buffer conditions, the mean migration time for thiourea was 4.11 minutes, with a mean electroosmotic flow mobility of 2.225 x 10^{-4} cm^2 $V^{-1}s^{-1}$. The migration times for benzoic acid, salicylic acid, and 3, 4, 5-trihydroxybenzoic acid were 4.71 minutes, 5.61 minutes and 6.94 minutes respectively as shown in Figure 2.13. Benzoic acid as the lowest molecular weight compound of the three, migrates fastest, while 3,4,5-trihydroxybenzoic acid migrates most slowly; this is in accordance with the fact that electrophoretic mobility is proportional to charge/mass ratio.

2.2.2 EOF study of basic compounds and their CE separation on fused silica capillary column using a normal polarity at pH 6.0

2.2.2.1 Introduction

The experiment was set to study EOF of background electrolyte and to analyse three bases (propanolol, pindolol and terbutaline) utilizing CE principle at a low pH condition in a normal polarity mode. The experiment also was performed to assure the use of CE instrument to analyse small molecules for our research.

2.2.2.2 Experimental

2.2.2.2.1 Chemicals and materials

Propanolol, pindolol, terbutaline, thiourea, sodium phosphate and sodium hydroxide were all purchased from Sigma-Aldrich (Dorset, UK). Fused silica capillary with a dimension of 100 µm (i.d.) x 375 µm (o.d.) was obtained from Composite Metal Services (Shipley, UK).

2.2.2.2.2 Instrumentation

Instrumentation used was as described in section 2.2.1.2.2.

2.2.2.2.3 Method

To study basic compounds using normal polarity CE at acidic pH (pH 6.0), a fused silica capillary of 45 cm total length, 36.5 cm effective length, and 100 µm (i.d.) x 375 µm (o.d.) was used. The BGE used was 25 mM sodium phosphate (pH 6.0). An applied voltage of +30 kV was used at a temperature of 25.0 °C, with UV detection at 210 nm. Injection was performed by using a pressure of 10 mBar for 5 seconds and applied potential at +30.0 kV using normal polarity mode.

Electroosmotic flow mobility (EOF) was determined by injecting thiourea at 30 µg/mL. The EOF value, effective mobility, and measured mobility of each compound were calculated using equations 13, 14 and 15. Each of the test compounds were diluted in the test mixture to the final concentration of 100 µg/mL in the BGE. Figure 2.14 shows the chemical structures of the test compounds propanolol, pindolol and terbutaline.

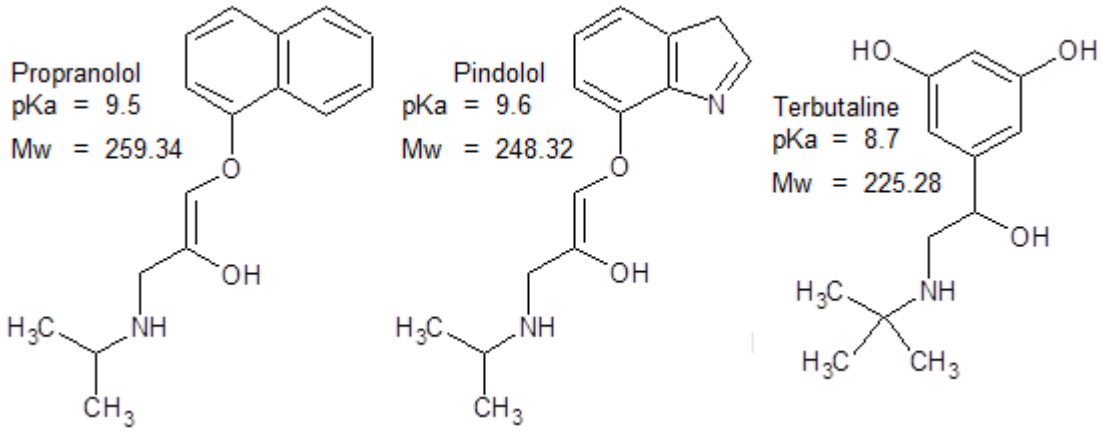


Figure 2.14 Chemical structures of basic test compounds propanolol, pindolol and terbutaline for a normal polarity CE

2.2.2.3 Results and discussion

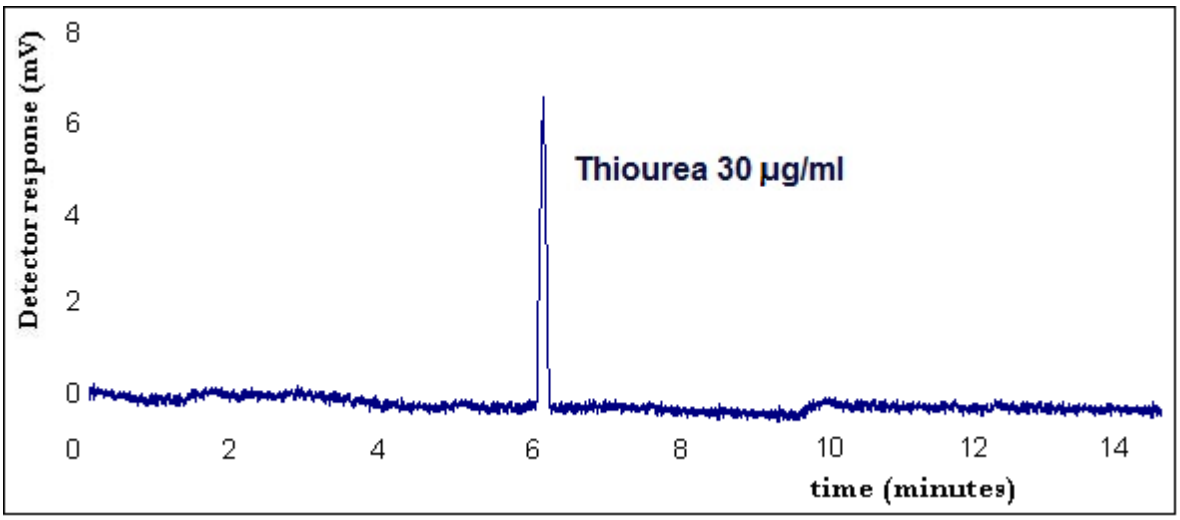


Figure 2.15 Electropherogram of thiourea (EOF marker) using a normal polarity CE with 25 mM sodium phosphate pH 6.0 as BGE, +30 kV applied voltage

The electropherogram in Figure 2.15 shows the EOF marker thiourea, as it appears when using a normal polarity CE, with BGE buffer at pH 6.0. For the normal polarity CE using a BGE of 25 mM sodium phosphate buffer (pH 6.0) and +30.0 kV applied voltage, basic compounds including propanolol, pindolol, and terbutaline were fully positively charged therefore they migrated from anode (+) to cathode (-) by the EOF value plus their electrophoretic mobility as shown in Table 7. The separation occurs because of the differences in their charge to mass ratios, since at pH 6.0 all of these analytes will be fully positively charged. The electroosmotic flow mobility (μ_{EOF}), effective mobility (μ_{eff}), and measured mobility (μ_{app}) are displayed in Table 2.7.

Table 2.7 Electrophoretic mobilities (x 10^{-4} cm^2 $V^{-1}s^{-1}$, absolute value) and migration times of propranolol, pindolol, and terbutaline at pH 6.0

Electropherogram number	EOF	Terbutaline			Pindolol			Propranolol		
	μ_{EOF}	t_m (min)	μ_{app}	μ_{eff}	t_m (min)	μ_{app}	μ_{eff}	t_m (min)	μ_{app}	μ_{eff}
1	1.491	7.90	1.155	0.336	8.40	1.086	0.405	9.49	0.962	0.529
2	1.550	7.86	1.161	0.389	8.37	1.090	0.460	9.44	0.967	0.583
3	1.555	7.89	1.157	0.398	8.39	1.088	0.467	9.46	0.965	0.590
4	1.549	7.85	1.162	0.387	8.38	1.089	0.460	9.43	0.968	0.582
5	1.563	7.87	1.159	0.403	8.36	1.092	0.471	9.42	0.969	0.594
6	1.545	7.86	1.161	0.384	8.34	1.094	0.450	9.40	0.971	0.574
MEAN	**1.542**	**7.87**	**1.159**	**0.383**	**8.37**	**1.090**	**0.452**	**9.44**	**0.967**	**0.575**
SD	**0.0257**	**0.0194**	**0.0029**	**0.0241**	**0.0216**	**0.0028**	**0.0243**	**0.0316**	**0.0032**	**0.0236**
%RSD	**1.67**	**0.25**	**0.25**	**6.29**	**0.26**	**0.26**	**5.38**	**0.33**	**0.33**	**4.09**

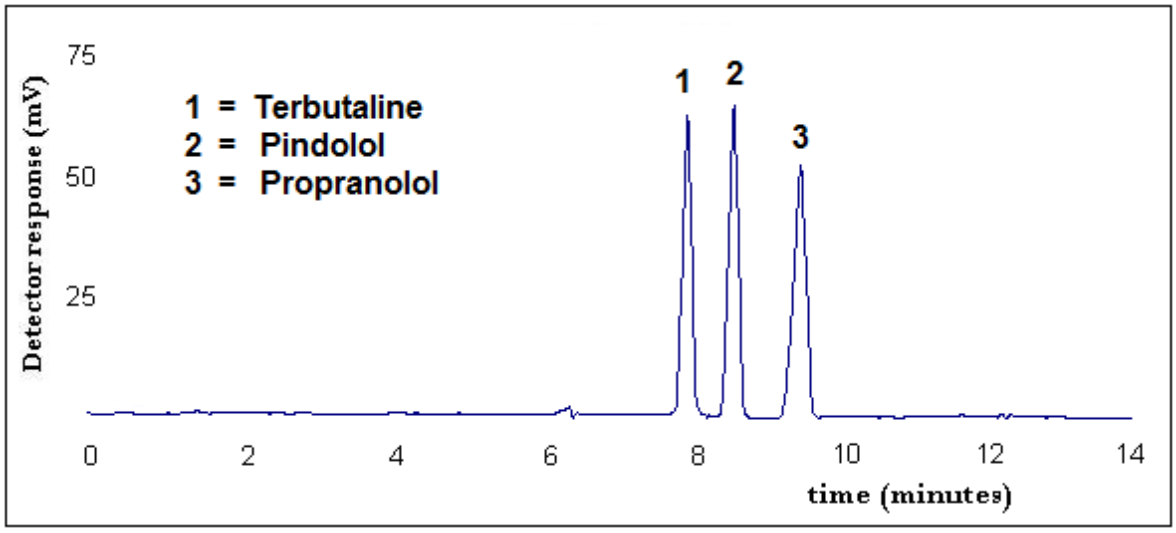


Figure 2.16 Electropherogram of terbutaline, pindolol and propranolol (basic compounds) using a normal polarity CE, with 25 mM sodium phosphate buffer (pH 6.0) as BGE and +30 kV applied voltage

Under the acidic pH (pH 6.0) conditions used, the mean migration time for thiourea was 6.12 minutes and provided mean electroosmotic flow mobility at 1.542 x 10^{-4} cm^2 $V^{-1}s^{-1}$. However, the average migration times for propranolol, pindolol, and terbutaline were 7.87 minutes, 8.37 minutes and 9.44 minutes respectively as shown in Figure 2.16.

Terbutaline and pindolol had a greater apparent mobility than propranolol because of their lower molecular weights. However, differences in the charge to mass ratios of these analytes affected their apparent mobilities, resulting in their separation in order of terbutaline, pindolol and propranolol respectively. It can be explained that at pH 6.0, terbutaline, pindolol and propranolol are all positively charged when running a normal polarity CEC. All positively charged molecules will migrate from anode to cathode because of their different electrophoretic mobilities. Regarding charge/ mass ratio, firstly terbutaline eluted, followed by pindolol, the second, and propranolol found the third because the molecular weight of terbutaline is least while the propranolol's is the most. Terbutaline will migrate fastest because of its smallest mass while propranolol will elute last because of its biggest mass.

Regarding results and findings from the micro-HPLC and the CE experiments, these micro-HPLC and CE instruments are capable to use for further experiments in order to suit our research.

Chapter 3

Reversed-phase with sulphonic acid groups monolithic column

Chapter 3. Reversed-phase with sulphonic acid groups monolithic column

In this chapter, a discussion of new reversed phase monoliths preparation to improve column performance on CEC, i.e., a long alkyl hydrocarbon polymer with sulphonic acid groups, pentaerythritol diacrylate monostearate (PEDAS-co-AMPS) was described. In previous study, Smith et al. [105] & Rassi and Bedair [106] fabricated PEDAS-co-AMPS columns by varying concentrations of AMPS in PEDAS monoliths on a variety of compounds. They found sulphonic acid groups of AMPS in the monoliths promoted electroosmotic flow for CEC analysis. It was proved that adding AMPS into the long alkyl hydrocarbon molecule for example PEDAS can be useful for our research and also can be further studied to fulfil our research gap.

Modified PEDAS-co-AMPS monolithic columns were prepared and studied in term of column efficiency using both micro-HPLC and CEC. The best performance column were compared with the monolithic HILIC column to suit our research on an application to small molecule analysis.

3.1 Fabrication of modified reversed-phase monoliths with sulphonic acid groups and their efficiency using micro-HPLC and CEC

3.1.1 Introduction

In this experiment, the research proposes to achieve better efficiency by using a modified reversed-phase monolithic column by adding sulphonic acid groups. The aim of this experiment is also to prove electroosmotic flow (EOF) from sulphonic moiety adding in monoliths providing a driving force for CEC separation [34]. Rassi

and Bedair [106] successfully reported and published using AMPS adding into the PEDAS monolith to generate an EOF driving force for CEC analysis. Also Smith et. al. [105] used the AMPS for fabrication of the poly (PEDAS-co-AMPS) to generate EOF for CEC. Three different percentages of adding of sulphonic acid groups into the reversed-phase monoliths were studied using both micro-HPLC and CEC to achieve the best performance column.

3.1.2 Experimental

3.1.2.1 Chemicals and materials

Pentaerythritol diacrylate monostearate (PEDAS), cyclohexanol, 2-acrylamido-2-methyl-1-propanesulphonic acid (AMPS), 2, 2'-azoisobutyronitrile (AIBN), 3-(trimethyoxysilyl) propyl methacrylate (γ-MAPS), TRIS (hydroxymethyl) aminomethane, ammonium hydroxide, acrylamide, toluene and thiourea were all purchased from Sigma-Aldrich (Dorset, UK) while HPLC-grade methanol and acetonitrile were obtained from Fisher Scientific (Leicestershire, UK). Deionised water was produced using a Millipore System (part no. SYNSV000). Ammonium formate was purchased from VWR International; Leicestershire, UK. Fused silica capillary with 100 µm (i.d.) x 375 µm (o.d.) was purchased from Composite Metal Services (Shipley, UK).

3.1.2.2 Instrumentation

Micro-HPLC experiments were carried out using a DiNa gradient pump, with a four port valco injection valve and a 100 nL internal loop. The UV detector was an Applied Biosystems 783 programmable absorbance detector with a capillary cell. Data acquisition and processing was carried out using DataApex Clarity

chromatography station. The oven used for the thermal polymerisation process was obtained from Genlab (Cheshire, UK). The microscope used during column preparation to check for presence of air bubbles inside the capillary was purchased from Jencons-Pls (model #00035160). The thermal stripper was sourced from Innova Tech (Stevenage, UK). The pH meter used during buffer pH adjustment was a Mettler Delta 320 meter, purchased from Mettler Toledo (Halstead, UK). Sonication was carried out using a XUBA3 ultrasonic bath, sourced from Grant Instruments (Cambridgeshire, UK). CEC were carried out on a Hewlett Packard 3DCE instrument (Waldbron, Germany).

3.1.2.3 Method

To synthesise and study the efficiency of the long alkyl chain monolith containing sulphonic acid groups, nine unmodified fused silica capillaries were pretreated by γ-MAPS. Three sets of capillaries were designed with 1%, 2% and 5% AMPS in the polymerisation mixtures. They were then filled and polymerised with the self polymerisable vinyl groups PEDAS mixture. Cyclohexanol, deionised water and ethylene glycol were used as porogenic solvents, with AIBN as an initiator for all mixtures. Table 3.1 lists the composition of the various mixtures, including the percentage (w/w) of porogen, monomer and initiators used in each case.

Table 3.1 Composition of polymerisation mixtures used for the preparation of a poly (PEDAS-co-AMPS) monolithic column

Column	Monomer mixture (% w/w)		Porogenic solvent (% w/w)			Polymerisation mixture (% w/w)		
	PEDAS	AMPS	Cyclohexanol	Ethylene glycol	Deionised water	Monomer	Porogen	Initiator
PA1	99	1	83.2	13.2	3.6	30	70	1
PA2	98	2	83.2	13.2	3.6	30	70	1
PA5	95	5	83.2	13.2	3.6	30	70	1

Three sets of columns (PA1, PA2 and PA5) were polymerised at 60 ºC in a Genlab oven overnight for 12 hours (n = 3). The polymerisation reaction of the long alkyl chain ligand (PEDAS) with a monomer containing sulphonic acid group (AMPS) is displayed in Figure 3.1.

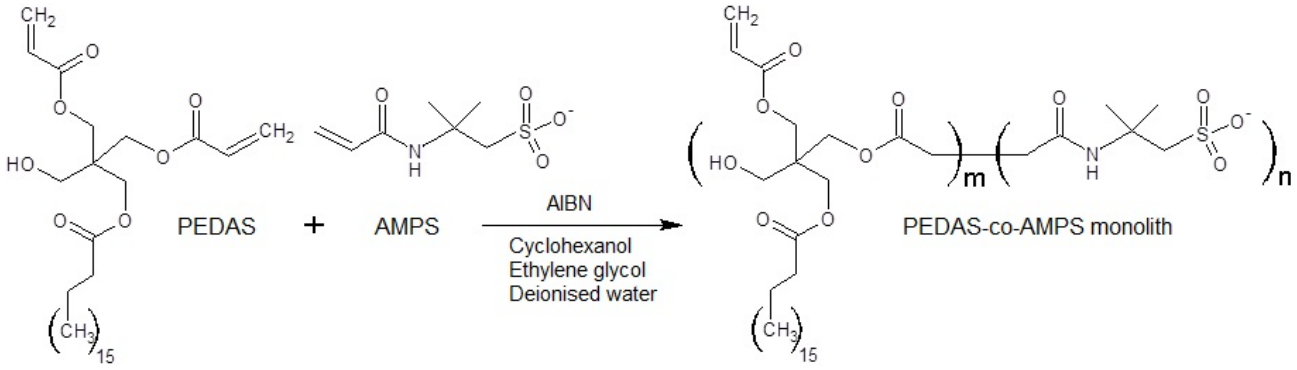


Figure 3.1 Polymerisation reaction for fabrication of poly (PEDAS-co-AMPS) monoliths

After completion of polymerisation, three sets of monolithic columns were washed out with methanol, followed by deionised water and then dried overnight by flushing with dry nitrogen gas. Three sets of capillaries were cut to a total length of about 39 cm with a desired effective length of about 30 cm. Next, a 2-3 mm detection window was created at a distance of 8.5 cm from the end of the column

using a thermal wire stripper. As a result, the monolithic polymers at this point were pyrolysed and flushed from the capillary with methanol, resulting a space for the detection window.

Four test compounds were injected under the following chromatographic conditions: a stock solution of 100 mM TRIS buffer pH 8.0. The mobile phase was prepared by mixing the desired amount of TRIS buffer pH 8.0 solution and acetonitrile (ACN). Test compound solutions were made by dilution of the sample with a 90: 10 (v/v) ACN: running buffer mixture, to provide a final concentration of 1 mg/mL. This was then diluted to the appropriate concentration of 100-200 µg/mL, depending on the sample. Finally, chromatograms from all sets were recorded and the numbers of the theoretical plates (*N*) for each column were calculated following Equation 4 from Chapter 1 and then compared to assess the relative efficiency of the columns.

3.1.3 Results and discussion

Results of the experiment including *N* values for three sets for each test compounds (anisole, dimethylphthalate, naphthalene and thiourea) and retention times are presented in Table 3.2. Example chromatograms obtained from the poly (PEDAS-co-AMPS) monolithic columns using 1%, 2% and 5% AMPS are shown in Figure 3.3. The chromatographic system for micro-HPLC in the experiment is as column; poly (PEDAS-co-AMPS) monolithic column, 300 mm x 100 µm (i.d.) x 375 µm (o.d.), mobile phase; ACN: 50 mM TRIS buffer pH 8.0 (90: 10), flow rate; 1000 nL/min, detection; UV at 214 nm, test compounds; anisole, dimethylphthalate, naphthalene, and thiourea and injection volume; 100 nL. The Figure 3.2 shows

chemical structures of test compounds (anisole, dimethylphthalate, naphthalene and thiourea.

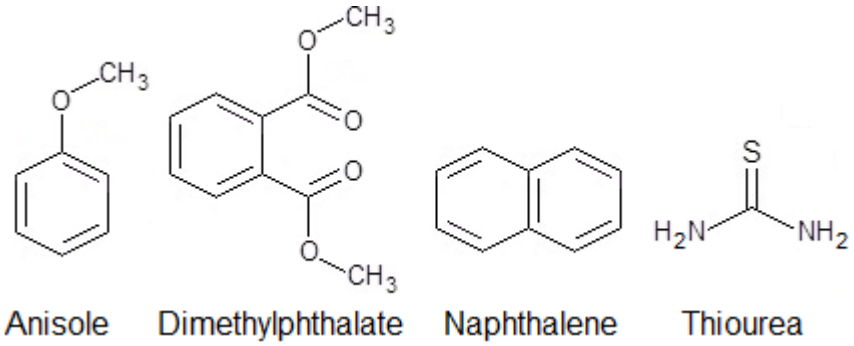
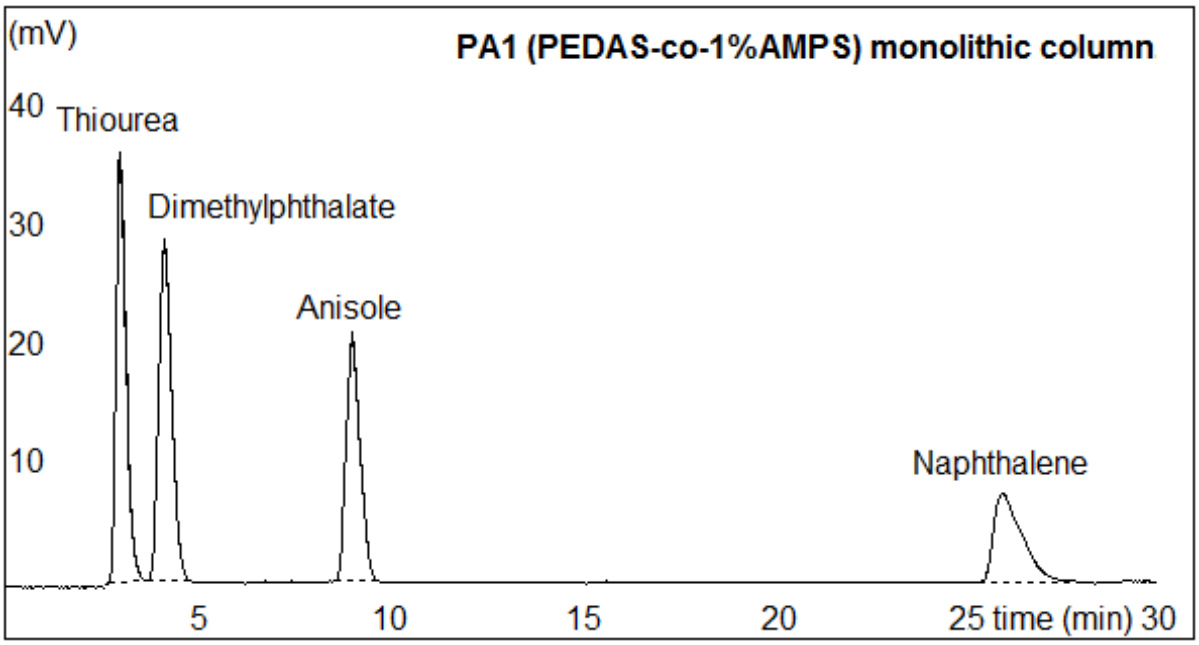


Figure 3.2 Chemical structures of anisole, dimethylphthalate, naphthalene, and thiourea

Table 3.2 Retention times and the numbers of the theoretical plates (*N*) for test analytes on monolithic columns with modified long alkyl chains and 1%, 2% and 5% sulphonic acid groups, poly (PEDAS-co-AMPS)

Column (%AMPS)	Thiourea	Dimethylphthalate	Anisole	Naphthalene
	N (per metre)/ [t_R (min)]	*N* (per metre)/ [t_R (min)]	*N* (per metre)/ [t_R (min)]	*N* (per metre)/ [t_R (min)]
PA1 (1.0)	7580.3 ± 75.8 [3.05 ± 0.05]	4077.0 ± 34.8 [3.97 ± 0.08]	6502.5 ± 22.6 [8.45 ± 0.11]	6303.2 ± 35.6 [25.95 ± 0.22]
PA2 (2.0)	8628.3 ± 93.7 [3.01 ± 0.04]	4694.7 ± 14.5 [3.84 ± 0.06]	7245.3 ± 12.4 [6.96 ± 0.08]	6986.7 ± 14.9 [18.27 ± 0.18]
PA5 (5.0)	7420.5 ± 91.7 [2.97 ± 0.05]	3814.9 ± 19.8 [3.75 ± 0.06]	6362.6 ± 17.1 [5.55 ± 0.09]	5971.9 ± 13.8 [12.31 ± 0.15]

Example chromatograms obtained from poly (PEDAS-co-AMPS) monolithic columns using 1%, 2% and 5% AMPS are shown in Figure 3.3.


(mV)
PA1 (PEDAS-co-1%AMPS) monolithic column
40
30
20
10
Thiourea
Dimethylphthalate
Anisole
Naphthalene
5
10
15
20
25 time (min) 30

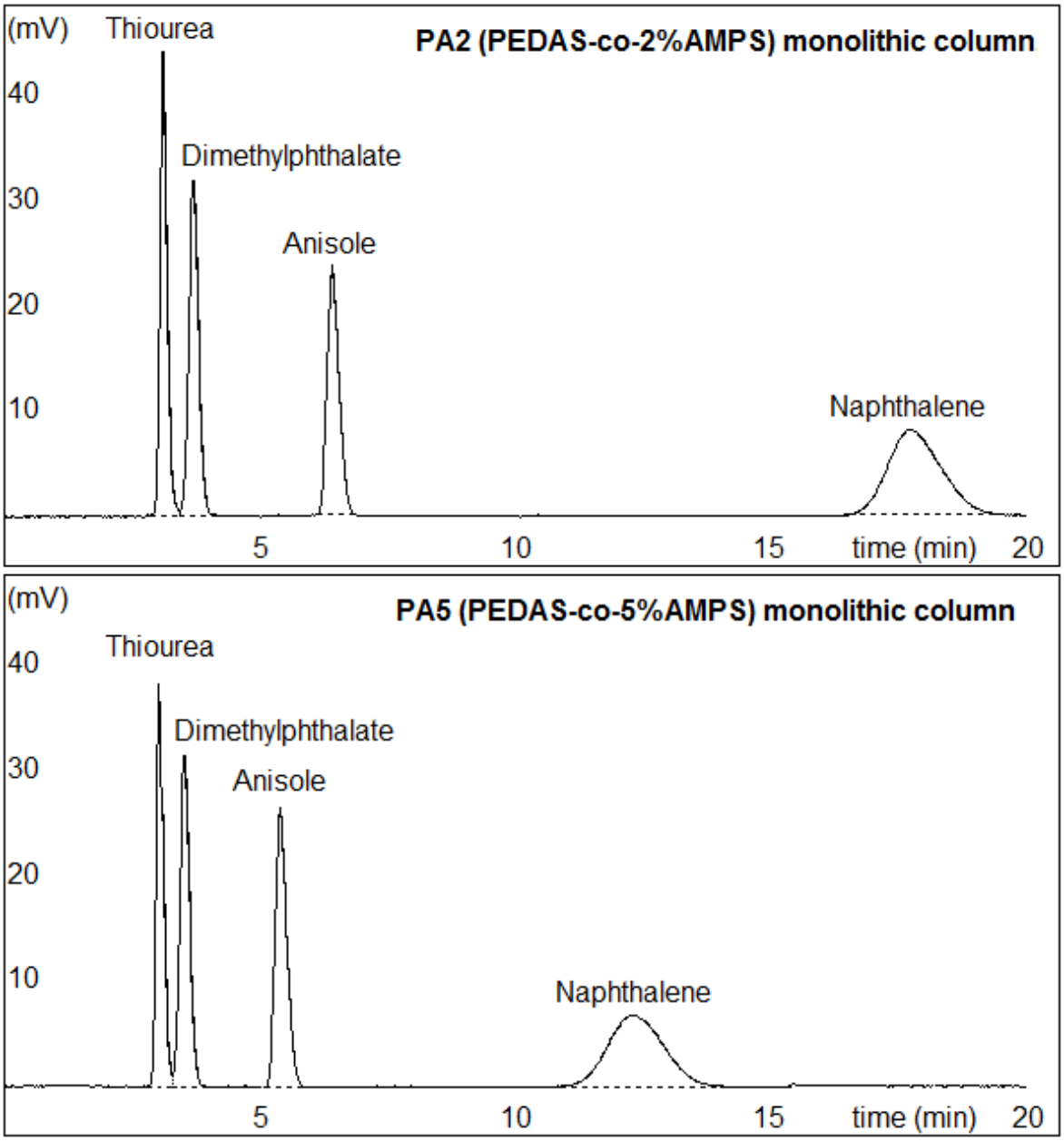


Figure 3.3 Micro-HPLC chromatograms of reversed-phase test compounds using three different modified reversed-phase monolithic columns (PEDAS-co-AMPS with 1%, 2% and 5% AMPS); mobile phase ACN: 50 mM TRIS buffer pH 8.0 (90: 10)

For all columns, the test compounds eluted in the order of decreasing polarity, as expected of the reversed-phase separation: thus, the most polar compound (thiourea) eluted first, dimethylphthalate second, anisole third and naphthalene last. Comparison of test compound efficiencies and retention times on the three monolithic columns (PEDAS-co-1% AMPS, PEDAS-co-2% AMPS and PEDAS-co-5% AMPS) showed that the lowest retention times were obtained at higher percentages of AMPS. This means the reversed-phase retention decreased with increasing sulphonic acid groups content. The PEDAS-co-5% AMPS monolithic column showed the fastest separation of test compounds, the PEDAS-co-1% AMPS showed the longest retention times under the same chromatographic conditions, and the PEDAS-co-2% AMPS gave the best column performance in terms of acceptable *N* values.

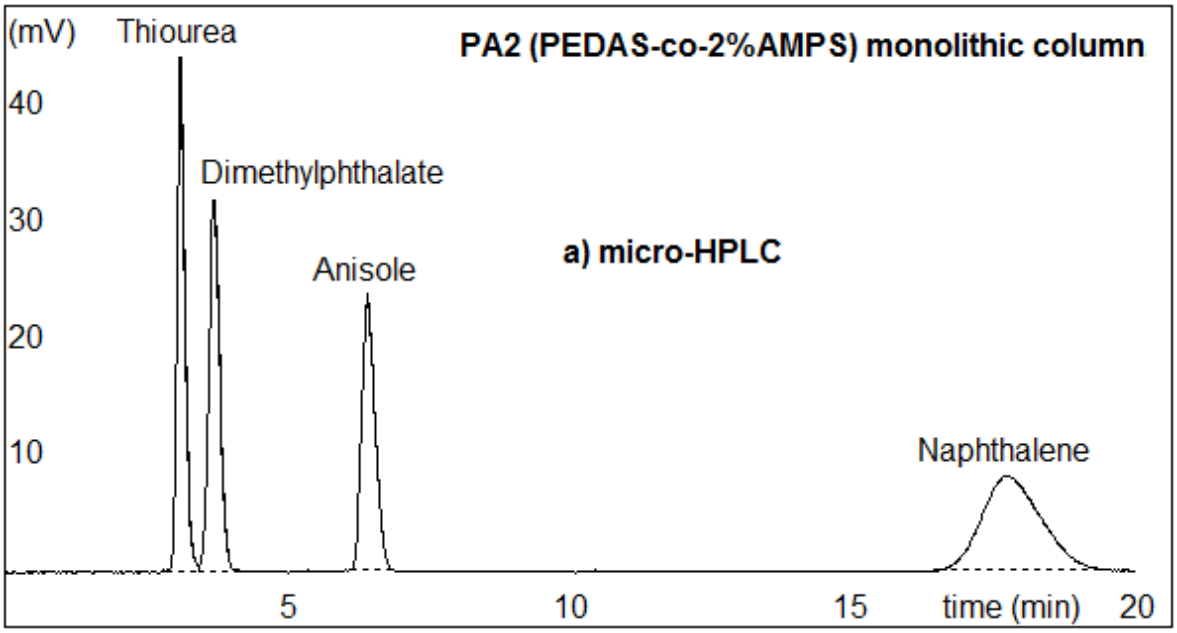
Accordingly, the PEDAS-co-2% AMPS monolithic column was selected to analyse the same set of test compounds using a CEC instrument under the same chromatographic conditions. A comparison of retention times and column performance on CEC and micro-HPLC for the PEDAS-co-2% AMPS monolith is shown in Table 3.3; example chromatograms for the same analyses are shown in Figure 3.4. CEC condition for the CEC experiment is as column; PEDAS-co-2% AMPS monolithic column 100 µm (i.d.) X 375 µm (o.d.) X 30.0 cm (effective length, 38.5 cm total length), background electrolyte (BGE); ACN: 50 mM TRIS buffer pH 8.0 (90:10), CEC mode; normal polarity, +30.0 kV, injection; 50 mBar X 1000 seconds, detection; UV 214 nm. CEC results comparison with micro-HPLC results

of the best modified long alkyl chain with sulphonic acid groups PEDAS-co-2% AMPS are shown in Table 3.3.

Table 3.3 Retention times and the numbers of the theoretical plates (*N*) of test compounds on the poly PEDAS-co-2% AMPS monolithic column using micro-HPLC and CEC

Method	AMPS (%)	Thiourea	Dimethylphthalate	Anisole	Naphthalene
		N (per metre)/ [t_R(min)]	*N* (per metre)/ [t_R(min)]	*N* (per metre)/ [t_R(min)]	*N* (per metre)/ [t_R(min)]
micro-HPLC	2.0	8628.3 ± 93.7 [3.01 ± 0.04]	4694.7 ± 14.5 [3.84 ± 0.06]	7245.3 ± 12.4 [6.96 ± 0.08]	6986.7 ± 14.9 [18.27 ± 0.18]
CEC	2.0	24432.1 ± 202.2 [6.18 ± 0.04]	27263.4 ± 670.3 [8.43 ± 0.08]	51797.3 ± 754.3 [15.99 ± 0.12]	58282.8 ± 880.9 [42.32 ± 0.22]

Example chromatograms obtained from poly PEDAS-co-2% AMPS monolithic columns using micro-HPLC and CEC are shown in Figure 3.4.



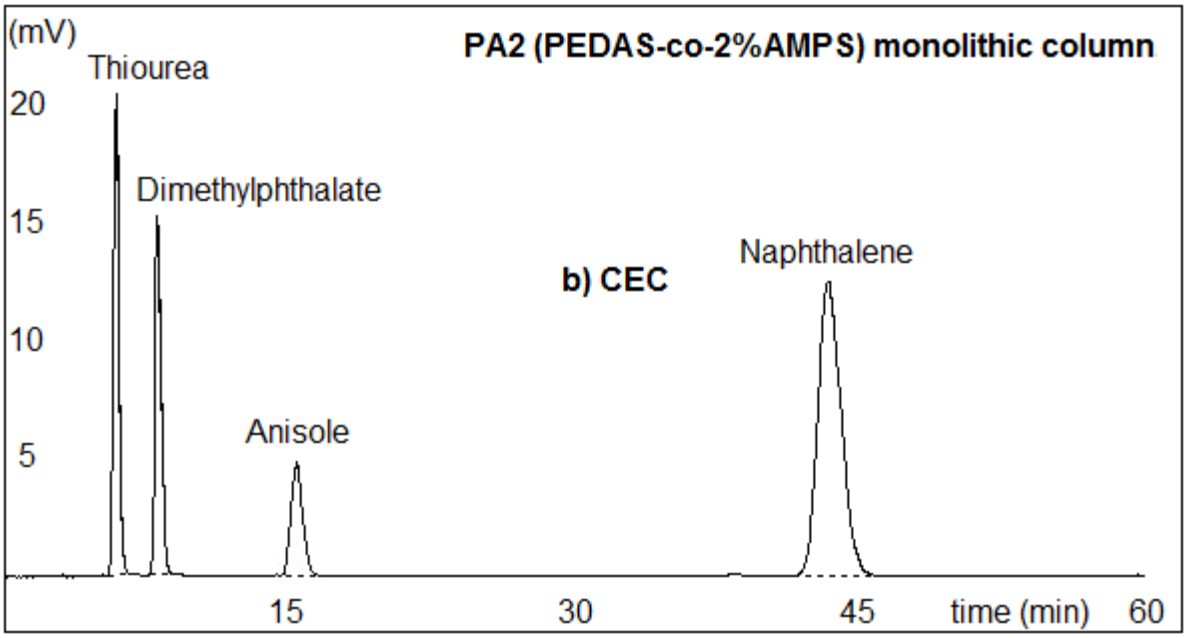


Figure 3.4 Separation of thiourea, dimethylphthalate, anisole and naphthalene in 90: 10 ACN: 50 mM TRIS buffer pH 8.0 on poly PEDAS-co-2% AMPS monolithic column by a) micro-HPLC and b) CEC

Generally, reversed-phase separations employ water and a miscible organic solvent (e.g. methanol, acetonitrile) in the mobile phase, but require much higher organic content in order to optimise the separation [95]. The separation mechanism of the long alkyl chain monolithic column modified with a sulphonic acid group is still the reversed-phase. Order of elution of test compounds followed the reversed-phase mechanism as observed in Table 3.3 and Figure 3.4. The poly (PEDAS-co-2% AMPS) monolithic column showed 3 to 8 times greater column efficiency on CEC than on micro-HPLC.

Referring to a) the micro-HPLC chromatogram and b) the CEC chromatogram in Figure 3.4, naphthalene as a nonpolar compound eluted after anisole, dimethylphalate and thiourea respectively when using the optimised condition. For

the polar compound thiourea, which was eluted first at high ACN content, opposite results from naphthalene were observed followed the reversed-phase mechanism of separation. These results demonstrated a typical reversed-phase retention mechanism only at the higher ACN content. It is quite interesting that on adding 2% AMPS to the RP monolith the efficiency in the CEC mode was greatly increased in line with theoretical predictions. These test compounds migrated from anode to cathode by the EOF resulting from the sulphonic moiety of the AMPS and the separation occurred as a result of a reversed-phase mechanism.

Furthermore, the modified reversed-phase (PEDAS) with 2% AMPS monolithic column can be applied to the separation of small non-polar molecules. Regard to both chromatograms (Figure 3.4 a) and b)), all peaks from a) micro-HPLC chromatogram are broader than those peaks from b) CEC. This phenomenon happened because of the fact that a beneficial plug-like driving force from CEC compared to micro-HPLC. A flow profile in a pressure driven system of micro-HPLC is parabolic, whereas an electrically driven system in CEC is a plug-like and therefore much more efficient. Consequently, this allows CEC to achieve very high efficiency. This experiment proved that the sulphonic groups from AMPS in the formed monoliths can generate an EOF to give a driving force for CEC separations for the further experiments.

Chapter 4

Modified HILIC with sulphonic acid groups monolithic column

Chapter 4. Modified HILIC with sulphonic acid groups monolithic column

It was proved that sulphonic acid groups inserting from AMPS molecules in monoliths can promote EOF for CEC separation in Chapter 3. In this study, adding AMPS in HILIC monolith structures proposes to get a benefit on driving forces which lead to CEC separation.

In this Chapter, a modified HILIC monolithic column with sulphonic acid groups was successfully fabricated and the column efficiency was evaluated using both micro-HPLC and CEC. The best performance column was compared with the monolithic HILIC column. Then the best column was delivered to apply on a small molecule analysis in Chapter 6.

4.1 Fabrication of HILIC and modified HILIC monoliths with sulphonic acid groups and their efficiency using micro-HPLC and CEC

4.1.1 Introduction

In this experiment, the aim was to achieve better efficiency using a modified HILIC (poly SPE-co-EDMA) monolithic column when adding sulphonic acid groups. As the study in Chapter 3 showed the electroosmotic flow (EOF) from sulphonic moiety added to monoliths can provide a driving force for CEC separation [34]. Rassi and Bediar [106] successfully presented AMPS adding into monoliths to give an EOF driving force for CEC analysis. Poly (SPE-co-EDMA), HILIC and modified poly (SPE-co-EDMA), HILIC with three different percentages of added sulphonic acid group monolithic columns were fabricated and studied using both micro-HPLC and CEC to achieve the best performance column.

4.1.2 Experimental

4.1.2.1 Chemicals and materials

All chemicals and materials were the same as those in the preparation of HILIC monolith experiment and AMPS (2-acrylamido-2-methyl-1-propanesulphonic acid were purchased from Sigma-Aldrich (Dorset, UK).

4.1.2.2 Instrumentation

The same as those in the HILIC monolith experiment

4.1.2.3 Method

To synthesis and study the efficiency of the HILIC, poly (SPE-co-EDMA) monolithic column and modified HILIC with sulphonic acid groups, poly (SPE-co-EDMA-co-AMPS) monolithic column. In this experiment, twelve unmodified fused silica capillaries (n = 3, for 4 sets) were derivatised with γ-MAPS and then polymerised using a mixture of SPE, EDMA and AMPS with methanol as a porogenic solvent and AIBN as an initiator. The proportions of monomers and porogen used are shown in Table 4.1.

Table 4.1 Composition of the polymerisation mixtures used for the preparation of poly (SPE-co-EDMA) and poly (SPE-co-EDMA-co-AMPS) monolithic columns

Column	Monomer mixture (%, w/w)			Porogen (%, w/w)	Polymerisation mixture (%, w/w)		
	SPE	EDMA cross-linker	AMPS EOF promotor	MeOH	Monomer mixture	Porogen solvent	Initiator, AIBN
SE	43.00	57.00	-	100.0	33.25	66.75	1.00
SEA1	42.57	56.43	1.00	100.0	33.25	66.75	1.00
SEA2	42.14	55.86	2.00	100.0	33.25	66.75	1.00
SEA5	40.85	54.15	5.00	100.0	33.25	66.75	1.00

All sets of HILIC columns were polymerised at 60 ºC in a Genlab oven overnight for 12 hours (n = 3). After finishing polymerisation, the set was prepared and all 3 test compounds were injected under the same chromatographic conditions (section 2.1.2.2.3.). All stock solutions of 5 mM ammonium formate, test compound solutions and all reagents were prepared as in the experiment in Chapter 3. The mobile phase was freshly prepared consisting of ACN: 5 mM ammonium formate pH 3.0 (90: 10 v/v). Finally, chromatograms from the twelve columns were then compared in terms of the efficiency of column. After the micro-HPLC experiment, the highest performance column was analysed by CEC with same test compounds then the efficiency compared with the results of micro-HPLC.

The polymerisation reaction of poly (SPE-co-EDMA-co-AMPS) monoliths is displayed in Figure 4.1.

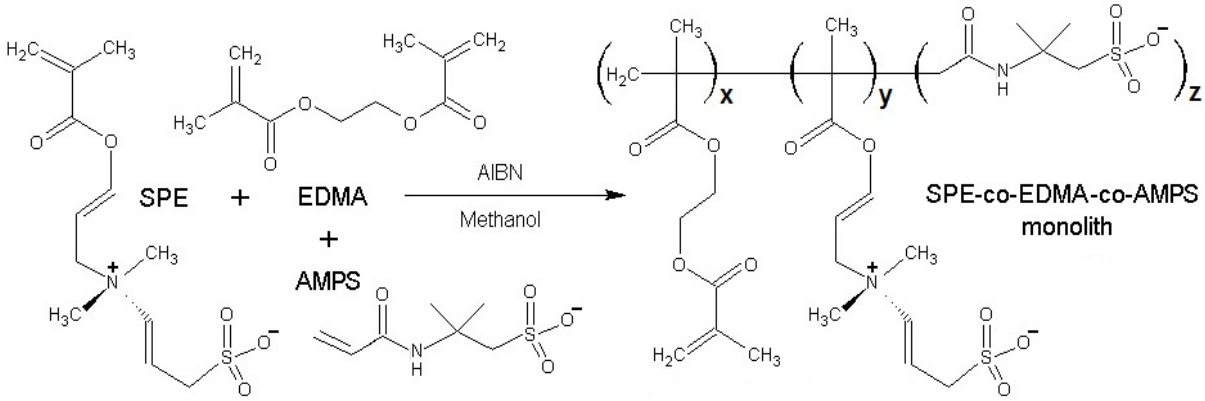


Figure 4.1 Fabrication of poly (SPE-co-EDMA-co-AMPS) monolithic polymerisation reaction

After the polymerisation was completed, all sets of monoliths were washed out with methanol, deionised water and dried overnight by flushing with dry nitrogen gas. Four sets of capillaries were cut to a total length of about 39 cm with a desired effective length of about 30 cm. Next, a 2-3 mm detection window was created at a distance of 8.5 cm from the end of the column using a thermal wire stripper. The monolithic polymers at this point were then pyrolysed and flushed from the capillary with methanol, providing a space for the detection window. The chromatographic condition for the experiment is as follow;

The mobile phase was prepared by mixing the desired amount of 5 mM ammonium formate solution pH 3.0 and ACN. Test compound solutions were made by dilution of the sample with ACN: running buffer (90: 10 v/v) to provide a final concentration of 1 mg/mL, then diluted to the appropriate concentration of 100-200 µg/mL. Finally, chromatograms from all sets were recorded and the numbers of the theoretical plates (*N*) were compared to assess the efficiency of the columns.

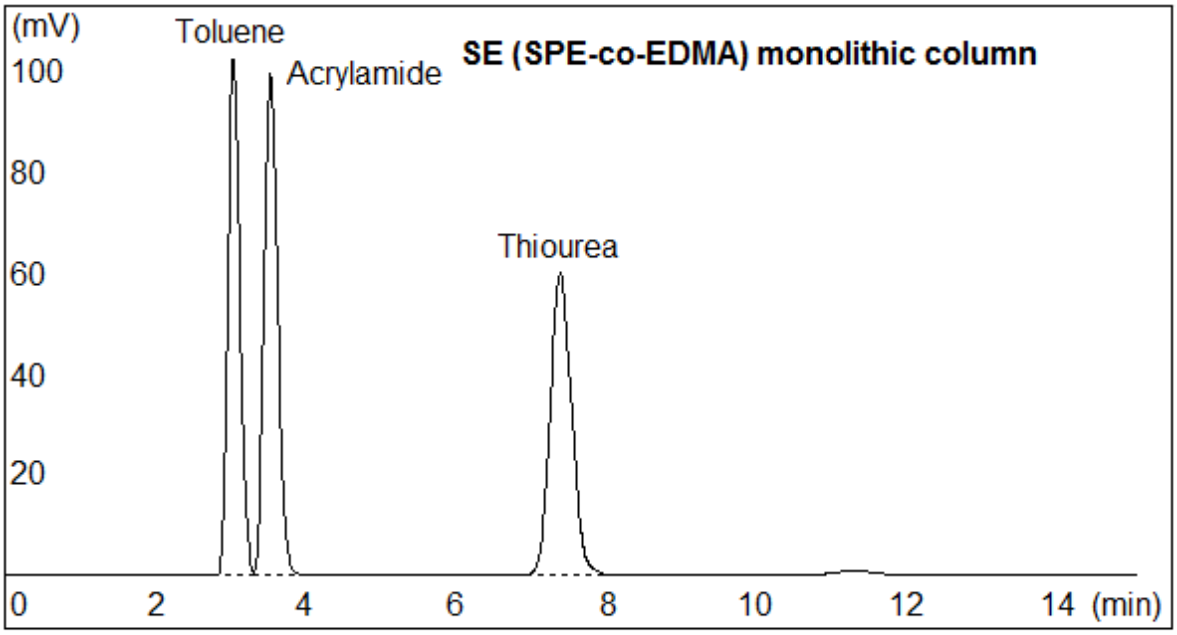
4.1.3 Results and discussion

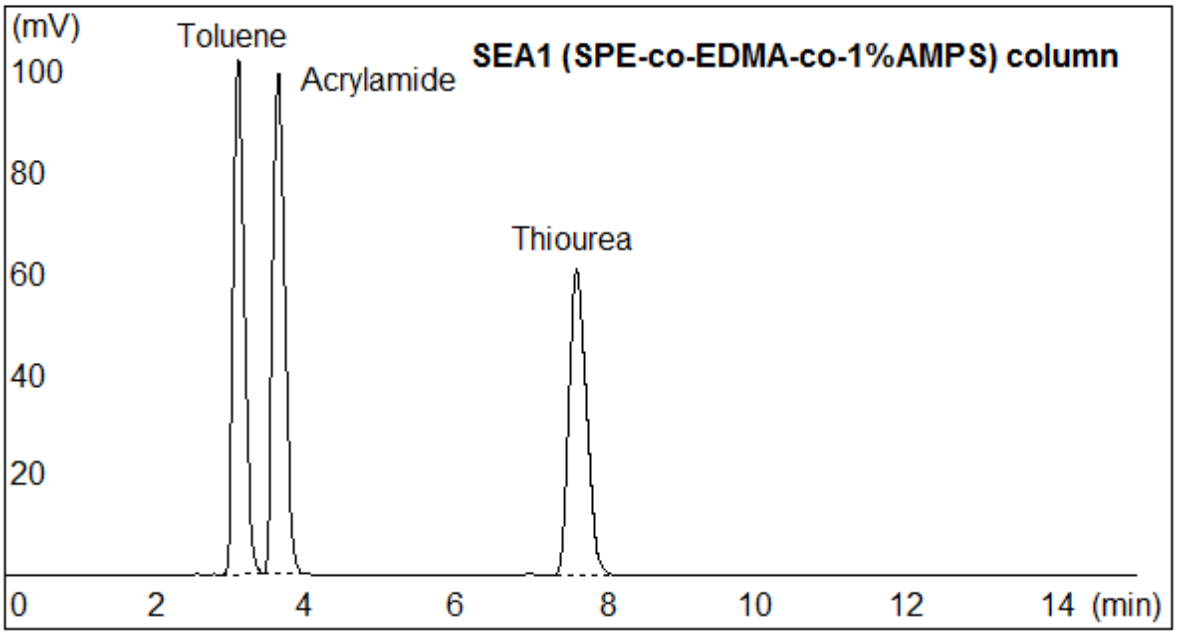
Results of the micro-HPLC experiment including the numbers of the theoretical plates (*N*) of four sets for each test compounds (toluene, acrylamide and thiourea) and retention times are presented in Table 4.2.

Table 4.2 List of test compounds, retention times, and the numbers of the theoretical plates (*N*) of HILIC, poly (SPE-co-EDMA) and modified HILIC with 1%, 2% and 5% sulphonic acid groups poly (SPE-co-EDMA-co-AMPS) monolithic columns

Column (%AMPS)	Toluene	Acrylamide	Thiourea
	N (per metre)/ [t_R (min)]	*N* (per metre)/ [t_R (min)]	*N* (per metre)/ [t_R (min)]
SE (-)	5452.1 ± 34.2 [3.02 ± 0.03]	5486.3 ± 28.6 [3.52 ± 0.04]	7768.9 ± 70.2 [7.33 ± 0.09]
SEA1 (1.0)	5997.2 ± 38.9 [3.09 ± 0.03]	7147.8 ± 33.9 [3.62 ± 0.05]	9504.8 ± 82.1 [7.55 ± 0.10]
SEA2 (2.0)	6547.3 ± 40.2 [3.21 ± 0.08]	9565.1 ± 51.9 [3.91 ± 0.09]	13841.1 ± 118.4 [8.91 ± 0.22]
SEA5 (5.0)	6298.9 ± 49.4 [3.68 ± 0.11]	8906.0 ± 58.6 [5.16 ± 0.14]	9553.1 ± 104.6 [12.18 ± 0.45]

Example chromatograms obtained from the poly HILIC, poly (SPE-co-EDMA) and poly modified HILIC, poly (SPE-co-EDMA-co-AMPS) monolithic columns using 1%, 2% and 5% AMPS are shown in Figure 4.2.


(mV)
100
80
60
40
20
Toluene
Acrylamide
Thiourea
SE (SPE-co-EDMA) monolithic column
0
2
4
6
8
10
12
14
(min)


(mV)
100
80
60
40
20
Toluene
Acrylamide
Thiourea
SEA1 (SPE-co-EDMA-co-1%AMPS) column
0
2
4
6
8
10
12
14
(min)

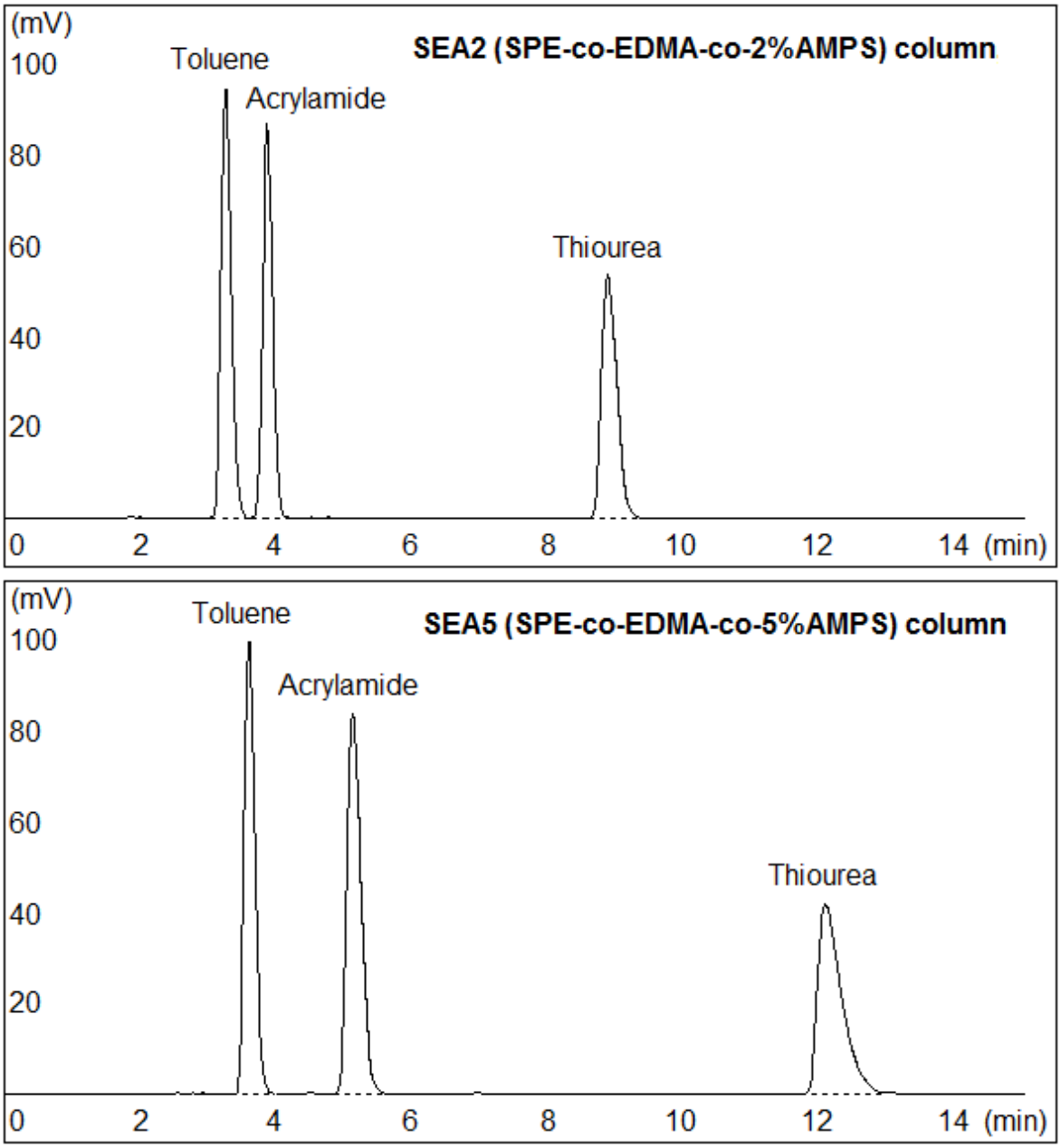


Figure 4.2 Micro-HPLC chromatograms of modified HILIC monoliths using three different percentages of sulphonic acid groups of poly (SPE-co-EDMA-co-AMPS with 1%, 2% and 5% AMPS) monolithic columns, mobile phase ACN: 5 mM ammonium formate pH 3.0 (90: 10)

Results from these experiments including the numbers of the theoretical plates (*N*) of each test compound (toluene, acrylamide and thiourea) and retention times using poly (SPE-co-EDMA-co-1% AMPS), poly (SPE-co-EDMA-co-2% AMPS) and poly (SPE-co-EDMA-co-5% AMPS) monolithic columns showed that the greater the percentage of polar AMPS in the monolith, resulted in more retention of the test compounds. This means that the HILIC retention increased with increasing sulphonic acid group content. The elution of test compounds followed the HILIC separation mechanism in the order of their increasing polarities. Toluene, the non-polar compound eluted first, followed by the neutral acrylamide was second, and then thiourea, the most polar compound eluted last.

Having the sulphonic acid groups (AMPS) in the HILIC monolith affected the partitioning mechanism of the solutes and stationary phase, the more of the AMPS polar moiety in the HILIC, the more polar the active site in the monolith. Therefore, thiourea as a polar compound retained in the column longer than acrylamide and toluene respectively. From the data in Table 4.2 and chromatograms in the Figure 4.2 the retention time of thiourea from poly (SPE-co-EDMA-co-5% AMPS) was highest at 12.18 minutes whereas the retention time of toluene was shortest at 3.02 minutes. The poly (SPE-co-EDMA-co-5% AMPS) monolithic column showed the slowest separation of test compounds under the same chromatographic conditions while the poly (SPE-co-EDMA) monolithic column provided the fastest separation. In terms of the numbers of the theoretical plates (*N*), the poly (SPE-co-EDMA-co-2% AMPS) gave the best column performance with high values of the

numbers of the theoretical plates (*N*) at 6547, 9565 and 13841 for toluene, acrylamide and thiourea respectively.

The best performance of the modified HILIC monolithic column with sulphonic acid groups (SPE-co-EDMA-co-2% AMPS) was selected to analyse the same test compounds using CEC under the same chromatographic conditions as follow, column; poly (SPE-co-EDMA-co-2% AMPS) monolithic column 100 µm (i.d.) X 375 µm (o.d.) X 30.0 cm (effective length), 38.5 cm (total length), BGE; ACN: 5 mM ammonium formate pH 3.0 (90: 10), CEC mode; normal polarity, +30.0 kV, Injection; 50 mBar X 1000 seconds, detection; UV 214 nm. The CEC results in comparison with the micro-HPLC results of the best modified HILIC monolithic column with sulphonic acid groups, poly (SPE-co-EDMA-co-2% AMPS) are shown in Table 4.3 and Figure 4.3.

Table 4.3 List of test compounds, retention times, and the numbers of the theoretical plates (*N*) of the modified HILIC monolith with 2% sulphonic acid groups, poly (SPE-co-EDMA-co-2% AMPS) monolithic column using micro-HPLC and CEC

Method	AMPS (%)	Toluene	Acrylamide	Thiourea
		N (per metre)/ [t_R(min)]	*N* (per metre)/ [t_R(min)]	*N* (per metre)/ [t_R(min)]
micro-HPLC	2.0	6547.3 ± 40.2 [3.21 ± 0.08]	9565.1 ± 51.9 [3.91 ± 0.09]	13841.1 ± 118.4 [8.91 ± 0.22]
CEC	2.0	40846.7 ± 1218.1 [5.19 ± 0.03]	51982.6 ± 1681.4 [5.98 ± 0.04]	62987.7 ± 1004.2 [12.07 ± 0.07]

Example chromatograms obtained from the poly (SPE-co-EDMA-co-2% AMPS) monolithic columns using micro-HPLC and CEC are shown in Figure 4.3.

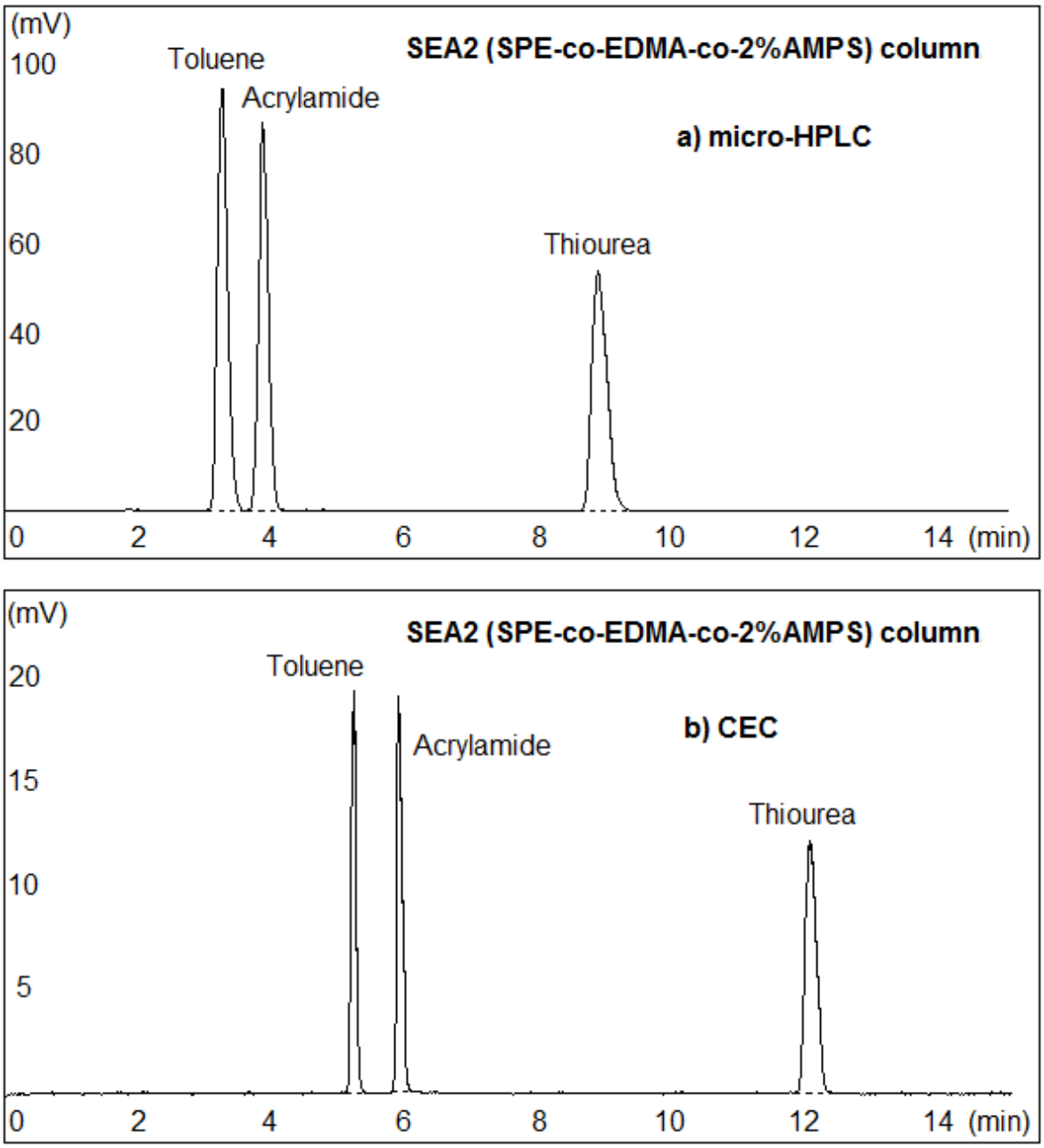


Figure 4.3 Separation of toluene, acrylamide and thiourea in ACN: 5 mM ammonium formate pH 3.0 (90: 10) on a poly (SPE-co-EDMA-co-2% AMPS) monolithic column by a) micro-HPLC and b) CEC techniques

Comparison of the micro-HPLC chromatogram and the CEC chromatogram in Figure 4.3, regarding the numbers of the theoretical plates (*N*), the best modified HILIC with sulphonic acid groups, poly (SPE-co-EDMA-co-2% AMPS) monolithic column showed 4 to 6 times greater column efficiency on CEC than micro-HPLC. In the CEC separation, the test compounds (toluene, acrylamide and thiourea) migrated from the anode to the cathode by the EOF promotion from the sulphonic acid moiety and the separation happened because of the HILIC mechanism. Furthermore, the modified HILIC, poly (SPE-co-EDMA) with 2% AMPS monolithic column can be applied for the separation of small molecules. This experiment proved the sulphonic acid groups from AMPS in monoliths can generate an EOF to give a driving force for CEC separations for the further experiments.

4.2 Manufacture of SPE-co-EDMA, SPE-co-EDMA-co-2% AMPS and SPE-co-EDMA with 10% particulate SCX monoliths: efficiency comparison using micro-HPLC and CEC techniques

4.2.1 Introduction

In this experiment, the study proposed to achieve the best performance column from three different modified organic HILIC columns as 1) a HILIC, poly (SPE-co-EDMA) monolithic column, 2) a modified HILIC, poly (SPE-co-EDMA) monolithic column with 2% AMPS and 3) a modified HILIC, poly (SPE-co-EDMA) with 10% particulate strong cationic exchange (SCX) monolithic column. As Experiment 4.1 showed the modified HILIC with 2% AMPS performed the highest the numbers of the theoretical plates *(N)* which proved that the electroosmotic flow (EOF) from sulphonic moiety can provide a driving force for CEC separation. The research proposed that having SCX in the monoliths will also give an EOF driving force for CEC analysis. Three types of these columns were prepared and evaluated using both micro-HPLC and CEC to achieve the best performance column. The most efficient column will be delivered to apply on an analysis of small molecules using both micro-HPLC and CEC in the Chapter 6.

4.2.2 Experimental

4.2.2.1 Chemicals and materials

All chemicals and materials were the same as those used in the preparation of HILIC monolith experiment, and AMPS (2-acrylamido-2-methyl-1-

propanesulphonic acid. Strong cationic exchange material (SCX) was given from Waters Ltd., UK.

4.2.2.2 Instrumentation

The same as those in the fabrication of modified reversed-phase monoliths experiment

4.2.2.3 Method

To synthesise and compare the efficiency of the HILIC, poly (SPE-co-EDMA) monoliths, modified HILIC with 2% sulphonic acid groups, poly (SPE-co-EDMA-co-2% AMPS) and HILIC with 10% particulate SCX monoliths, nine unmodified fused silica capillaries (n = 3, three sets) were pretreated with γ-MAPS and then polymerised by using the SPE, EDMA, AMPS and SCX material (3 µm diameter) mixtures with methanol as a porogenic solvent and AIBN as an initiator. The 10% by weight SCX material was included in the monomer mixture so that the SCX was distributed throughout the monolith. The proportions of monomers, EOF promotors and porogen used are shown in Table 4.4.

Table 4.4 List of compositions of polymerisation mixtures used for preparation of poly (SPE-co-EDMA), poly (SPE-co-EDMA-co-2% AMPS) and poly (SPE-co-EDMA with 10% particulate SCX) monolithic columns

Column	Monomer mixture (%, w/w)				Porogen (%, w/w)	Polymerisation mixture (%, w/w)		
	SPE	EDMA cross-linker	AMPS EOF promoter	SCX EOF promoter	MeOH	Monomer mixture	Porogen solvent	Initiator, AIBN
SE	43.00	57.00	-	-	100.0	33.25	66.75	1.00
SEA2	42.14	55.86	2.00	-	100.0	33.25	66.75	1.00
SEC10	38.70	51.30	-	10.0	100.0	33.25	66.75	1.00

All sets of HILIC columns were polymerised at 60 ºC in a Genlab oven overnight for 12 hours (n = 3). After finishing polymerisation, the set was prepared and all 3 test compounds were injected under the same chromatographic conditions (section 2.1.2.2.3). All stock solutions of 5 mM ammonium formate, test compound solutions and all reagents were prepared as in the previous experiment. The mobile phase was freshly prepared consisting of ACN: 5 mM ammonium formate pH 3.0 (90: 10). Finally, chromatograms from nine columns were then compared in terms of the efficiency of column. After the micro-HPLC experiment, the best performance column was selected for further experiment.

In addition, the polymerisation reaction of SPE-co-EDMA monolith with Strong Cationic Exchange particle is displayed in Figure 4.4.

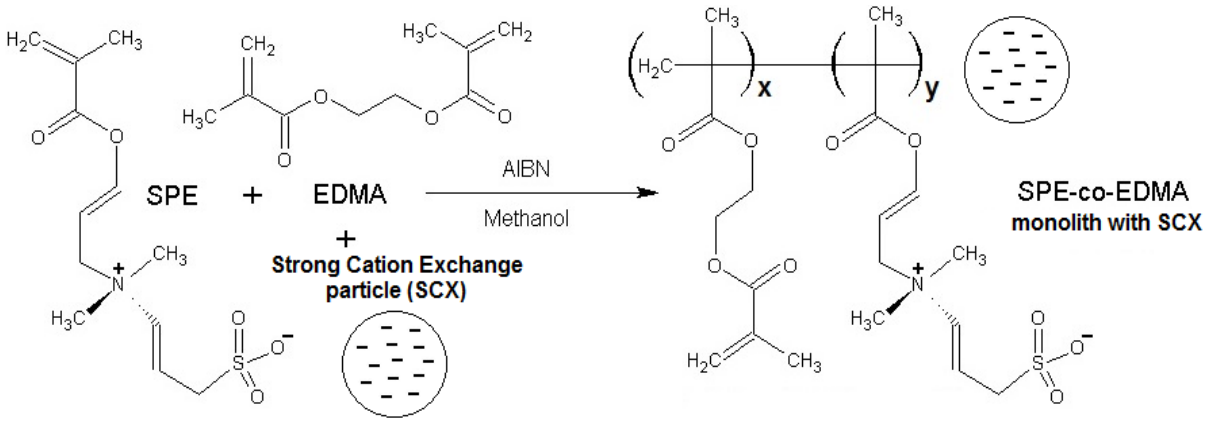


Figure 4.4 Fabrication of HILIC, poly (SPE-co-EDMA) monolithic polymerisation reaction with Strong Cationic Exchange (SCX) particle

After the polymerisation was completed, all sets of monoliths were washed out with methanol, deionised water and dried overnight by flushing with dry nitrogen gas. All sets of capillaries were cut to a total length of about 39 cm with the desired effective length about 30 cm then 2-3 mm detection windows were created at a

distance of 8.5 cm from the end of the column using a thermal wire stripper. Monolithic material at this point was pyrolysed and then flushed out with methanol forming the clear detection window for the column. All columns were then washed with ACN and were used for the experiment. The columns were equilibrated with ACN: 5 mM ammonium formate pH 3.0 (90: 10) then they were evaluated using micro-HPLC as a chromatographic system, columns; 1) a HILIC, poly (SPE-co-EDMA), SE monolithic column, 2) a modified HILIC, poly (SPE-co-EDMA) with 2% AMPS monolithic column, SEA2 and 3) a modified HILIC, poly (SPE-co-EDMA with 10% particulate strong cationic exchange, SCX), SEC10 monolithic columns; 100 µm (i.d.) X 375 µm (o.d.) X 300 mm column (n = 3), mobile phase; ACN: 5 mM ammonium formate pH 3.0 (90: 10 and 97.5: 2.5), flow rate; 1000 nL/min, injection volume; 100 nL, detection; UV 214 nm, test compounds; toluene, acrylamide, thiourea 100 µg/ml. Finally, chromatograms from three columns were then compared in terms of the efficiency of columns (the numbers of the theoretical plates, *N*).

After the micro-HPLC experiment, all columns were equilibrated with the same mobile phase then analysed by CEC using the same test compounds then compared the efficiency in terms of the numbers of the theoretical plates (*N*) with the results of micro-HPLC. CEC condition for the CEC experiment was as column; 1) a HILIC, poly (SPE-co-EDMA) monolithic column, 2) a modified HILIC, poly (SPE-co-EDMA) monolithic column with 2% AMPS and 3) a modified HILIC, poly (SPE-co-EDMA) with 10% particulate strong cationic exchange (SCX) monolithic column; 100 µm (i.d.) X 375 µm (o.d.) X 300 mm column (n = 3), (30.0 cm effective

length, 38.5 cm total length), background electrolyte (BGE); ACN: 5 mM ammonium formate pH 3.0 (97.5: 2.5), CEC mode; normal polarity, +30.0 kV, injection; 50 mBar X 1000 seconds, detection; UV 214 nm.

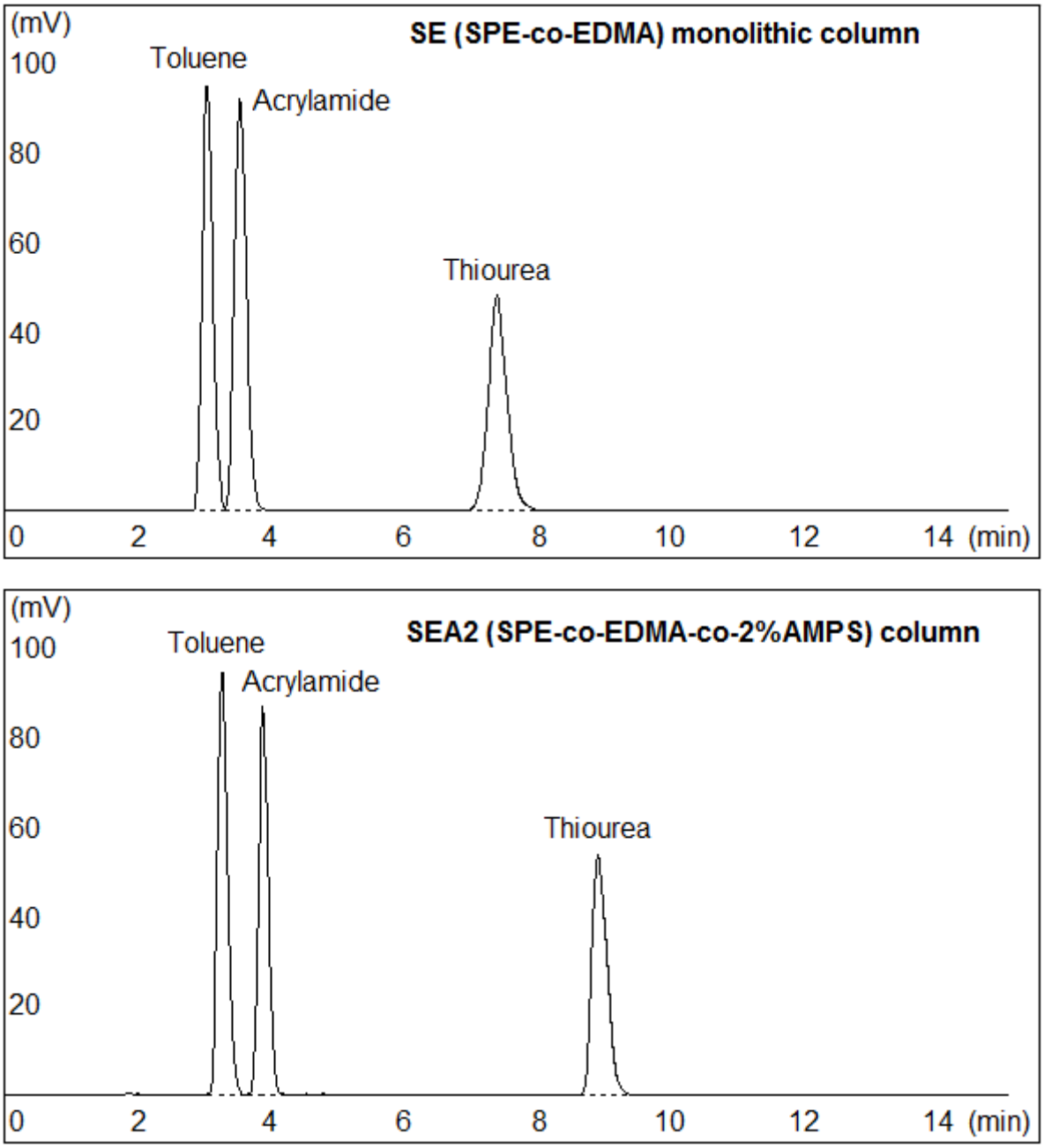
4.2.3 Results and discussion

Results of the micro-HPLC experiment including the numbers of theoretical plates (*N*) of three sets for each of the test compounds (toluene, acrylamide and thiourea) and retention times are presented in Table 4.5.

Table 4.5 List of test compounds, retention times, and the numbers of the theoretical plates (*N*) from the HILIC, poly (SPE-co-EDMA) monolith, modified with AMPS, poly (SPE-co-EDMA-co-2% AMPS) and the HILIC monolith containing 3 µm SCX material, poly (SPE-co-EDMA with 10% SCX) monolithic columns with mobile phase of ACN: 5 mM ammonium formate pH 3.0 (90: 10) and (97.5: 2.5)

Column (ACN:buffer pH3)	% EOF Promotor	Toluene	Acrylamide	Thiourea
		N (per metre)/ [t_R (min)]	*N* (per metre)/ [t_R (min)]	*N* (per metre)/ [t_R (min)]
SE (90:10)	-	5378.1 ± 28.9 [3.01 ± 0.04]	5514.2 ± 31.2 [3.55 ± 0.05]	7753.7 ± 64.8 [7.37 ± 0.09]
SEA2 (90:10)	2.0 (AMPS)	6602.1 ± 45.3 [3.16 ± 0.06]	9539.3 ± 69.2 [3.98 ± 0.08]	14021.4 ± 127.9 [8.99 ± 0.27]
SEC10 (90:10)	10.0 (SCX)	1419.3 ± 19.1 [2.14 ± 0.03]	1418.4 ± 16.5 [2.43 ± 0.03]	1931.5 ± 21.2 [4.93 ± 0.05]
SEC10 (97.5:2.5)	10.0 (SCX)	1565.2 ± 20.5 [2.13 ± 0.03]	1607.6 ± 21.8 [3.04 ± 0.03]	1470.8 ± 28.9 [16.07 ± 0.39]

Example chromatograms obtained from the three types columns using a mobile phase of ACN: 5 mM ammonium formate pH 3.0 (90: 10) for SE, SEA2, SEC10 and (97.5: 2.5) for SEC10 are respectively shown in Figure 4.5.



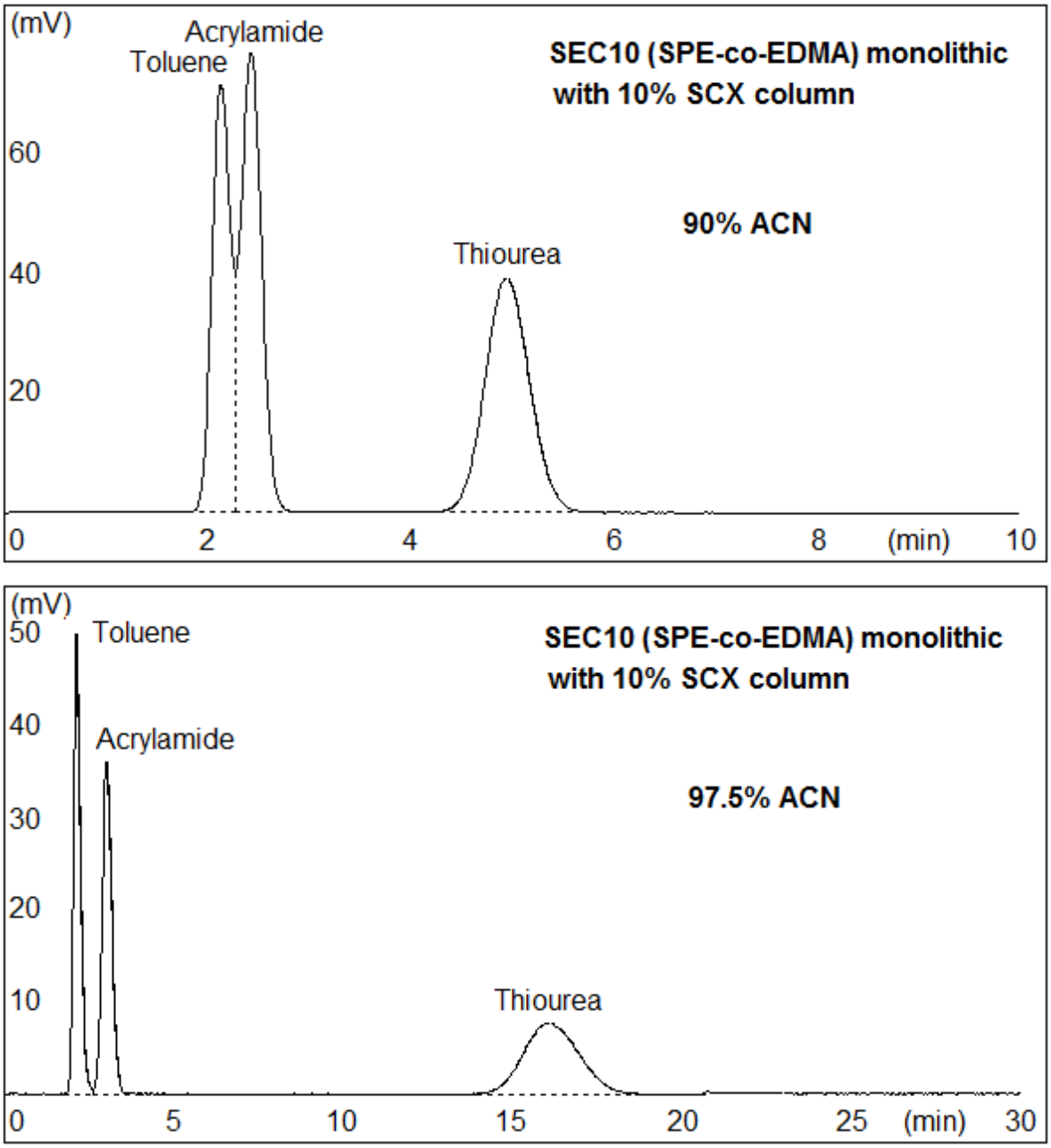


Figure 4.5 Micro-HPLC chromatograms of modified HILIC test compounds using poly (SPE-co-EDMA), poly (SPE-co-EDMA-co-2% AMPS), and poly (SPE-co-EDMA monolith with 10% SCX) monolithic columns using mobile phase of ACN: 5 mM ammonium formate pH 3.0 (90: 10) and (97.2: 2.5)

Results of the experiment including the numbers of the theoretical plates (*N*) of each of the test compounds (toluene, acrylamide and thiourea) and retention times using poly (SPE-co-EDMA) and poly (SPE-co-EDMA-co-2% AMPS) monolithic columns confirmed the previous result of HILIC retention property that adding the polar moiety (sulphonic acid groups) via 2% AMPS into the HILIC (SPE-co-EDMA) monolith could increase HILIC retention with improving efficiency and performance of the column.

Regarding the HILIC monolith (SPE-co-EDMA) with 10% (3 µm) SCX, it was found that on adding the strong cationic exchange material into the HILIC monolith, the HILIC retention was significantly decreased with unacceptable column performance using the same mobile phase conditions (ACN: 5 mM ammonium formate pH 3.0; 90: 10). The test compounds separation was optimised by increasing the acetonitrile content from 90 to 97.5 as presented in the chromatogram. The results from the experiment confirmed that the modified HILIC monolith with sulphonic acid groups (SPE-co-EDMA-co-2% AMPS) was the best performing column and this monolith was therefore selected to analyse the same test compounds and also applied to small basic compounds analysis using micro-HPLC and CEC instruments.

The SEC10 monolithic column was tested with CEC chromatographic system with 97.5% ACN background electrolyte to achieve the best result. An example of CEC chromatogram of the modified HILIC monolith with 10% SCX materials is presented in Figure 4.6.

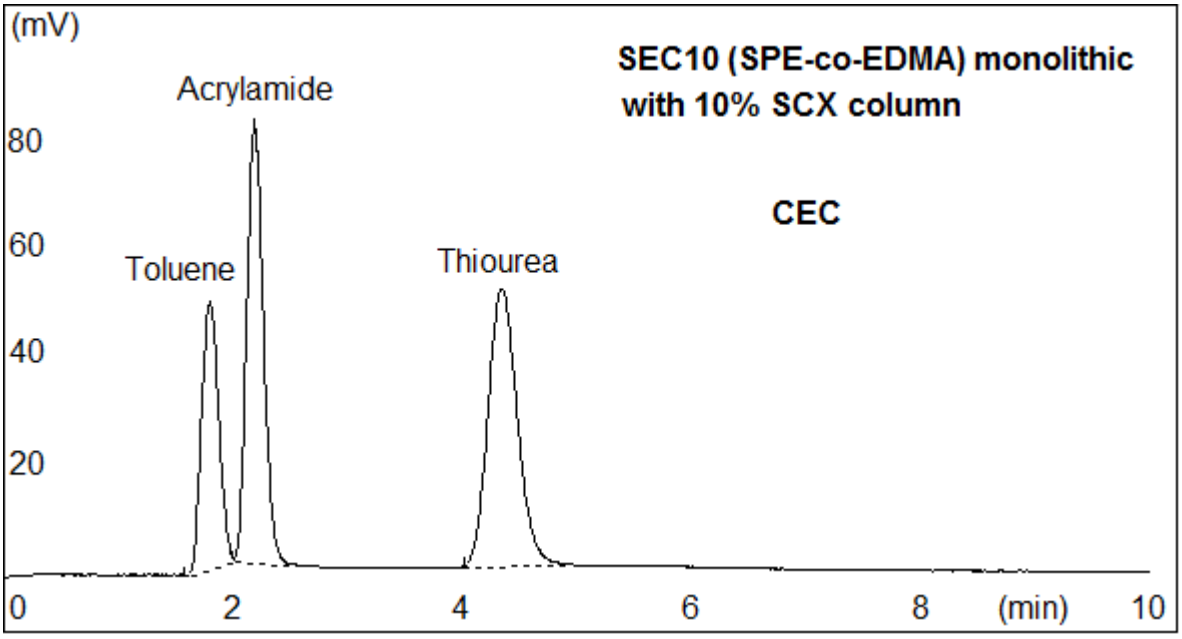


Figure 4.6 A separation of toluene, acrylamide and thiourea in ACN: 5 mM ammonium formate pH 3.0 (97.5: 2.5) on poly (SPE-co-EDMA with 10% particulate SCX (SEA10)) monolithic column by CEC

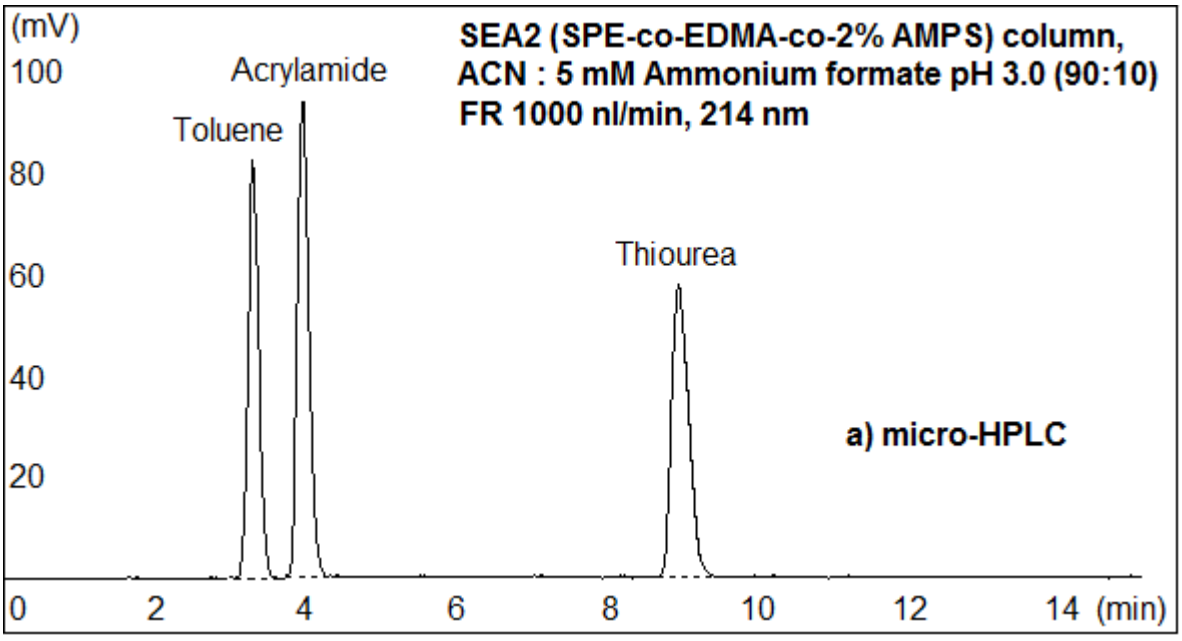
As a CEC chromatogram shown in Figure 4.6, the column performance of the optimised condition on a poly (SPE-co-EDMA with 10% particulate SCX (SEA10)) column is still poor when compare with the modified HILIC with sulphonic acid groups a poly (SPE-co-EDMA-co-2% AMPS, SEA2) column. Therefore the SEA2 was still considered to be the best column and used for further experiment.

Comparisons of CEC results with micro-HPLC results of the best modified HILIC with sulphonic acid groups a poly (SPE-co-EDMA-co-2% AMPS) monolithic column are shown in Table 4.6 and Figure 4.7.

Table 4.6 List of test compounds, retention times, and the numbers of the theoretical plates (*N*) of modified HILIC with 2% sulphonic acid groups, a poly (SPE-co-EDMA-co-2% AMPS) monolithic column using micro-HPLC and CEC

Method	AMPS (%)	Toluene	Acrylamide	Thiourea
		N (per metre)/ [t_R (min)]	*N* (per metre)/ [t_R (min)]	*N* (per metre)/ [t_R (min)]
micro-HPLC	**2.0**	6602.1 ± 45.3 [3.16 ± 0.06]	9539.3 ± 69.2 [3.98 ± 0.08]	14021.4 ± 127.9 [8.99 ± 0.27]
CEC	**2.0**	40754.6 ± 1194.4 [5.15 ± 0.04]	52097.5 ± 1663.7 [5.95 ± 0.05]	63116.9 ± 1194.7 [12.15 ± 0.09]

Example chromatograms obtained from the poly (SPE-co-EDMA-co-2% AMPS) monolithic column using micro-HPLC and CEC are shown in Figure 4.7.



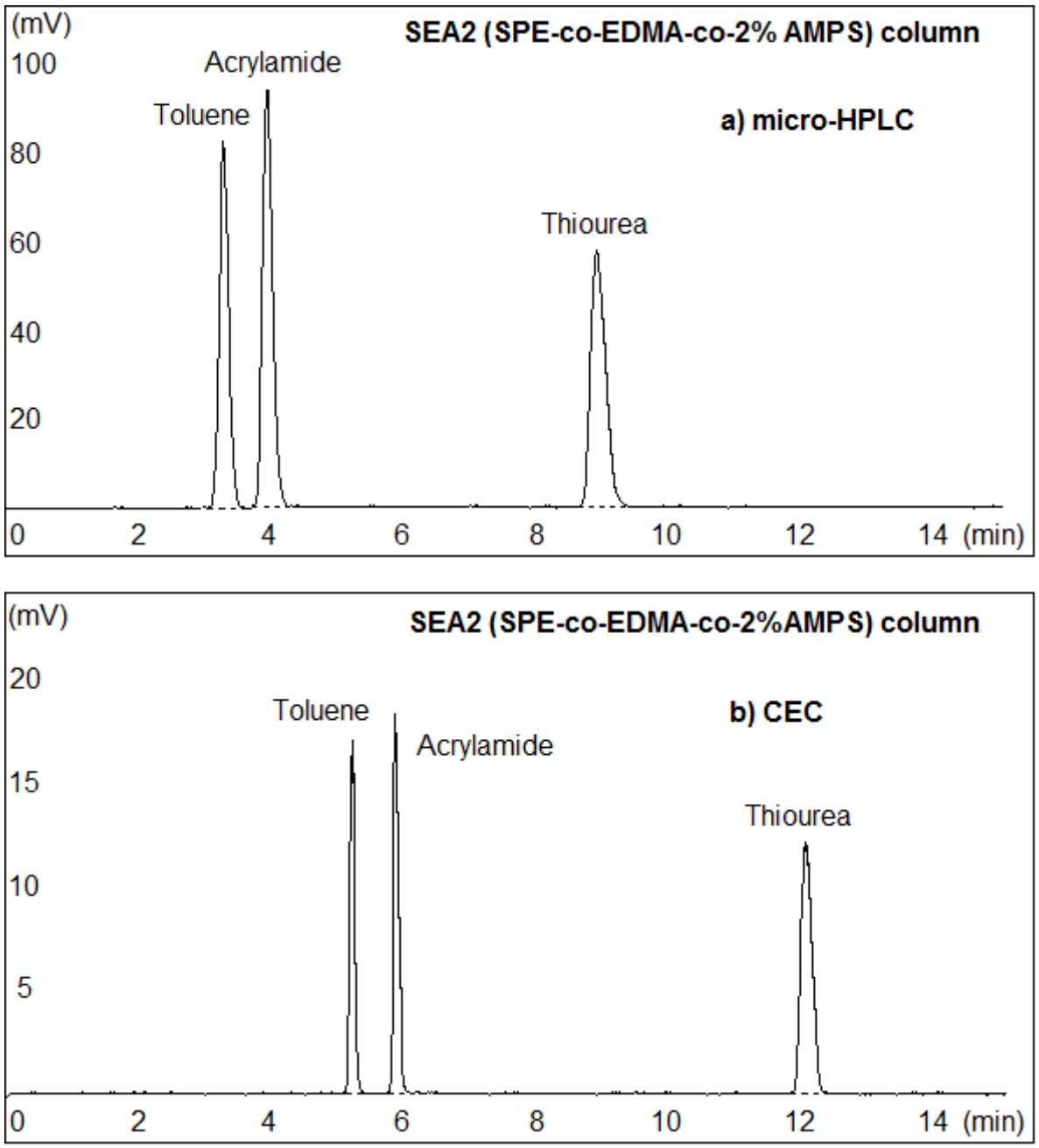


Figure 4.7 Separation of toluene, acrylamide and thiourea in ACN: 5 mM ammonium formate pH 3.0 (90: 10) on a poly (SPE-co-EDMA-co-2% AMPS) monolithic column by a) micro-HPLC and b) CEC techniques

Referring to a) the micro-HPLC chromatogram and b) the CEC chromatogram in Figure 4.7, and regarding the numbers of the theoretical plates (*N*) from Table 4.6,

the best modified HILIC with sulphonic acid groups, poly (SPE-co-EDMA-co-2% AMPS) monolithic column showed 4.5 to 6 times greater column efficiency on CEC than micro-HPLC. CEC theory predicts greater efficiency than pressure driven separations due among other things to the laminar flow profile generated by electrically driven flow, compared to the parabolic flow profile generated from pressure driven systems.

In the CEC separation (b) these test compounds (toluene, acrylamide and thiourea) migrated from anode to cathode by the EOF promotion from the sulphonic acid moiety of the AMPS and the separation happened because of the HILIC mechanism. Furthermore, the modified HILIC, poly (SPE-co-EDMA with 2% AMPS) monolithic column can be applied for the separation of small molecules. This experiment proved the sulphonic acid groups from AMPS in the monoliths can generate EOF to give a driving force for CEC separation for the further experiments.

Chapter 5

Commercial packed HILIC column

Chapter 5. Commercial packed HILIC columns

Commercial packed HILIC columns have been developed and used for analysis of pharmaceutical compounds for decades. It is interesting that mixing CEC promoter particles with HILIC stationary phase materials may increase column efficiency and may enhance sensitivity of detection for CEC analysis.

Moreover, EOF enhancement on the study was also demonstrated in the chapter. It focuses on the development of packed commercial HILIC material with commercial strong cationic exchange (SCX) stationary phase for CEC. Mixed-mode packed HILIC with SCX columns were prepared and evaluated using both micro-HPLC and CEC techniques.

In this Chapter, the manufacture of a HILIC packed stationary phase mixed with a phase containing sulphonic acid groups was performed in order to generate a driving force for CEC separations. Then an efficiency performance of commercial packed HILIC column was determined and then compared with the best performance column from our previous experiments (a modified HILIC with AMPS monolithic column). Finally, the best performance column was then used on a small molecule analysis for the research.

5.1 Manufacture of a commercial HILIC phase mixed with commercial SCX materials and efficiency comparison between micro-HPLC and CEC techniques

5.1.1 Introduction

In this study, the efficiency of the Atlantis HILIC packing materials combined with Strong Cationic Exchange (SCX) materials using micro-HPLC and CEC was investigated. Having a cationic moiety from SCX in the HILIC stationary phase will affect HILIC separation mechanism which makes the stationary phase more polar. Moreover, it also generates an EOF driving force when applying to CEC. The aim for this chapter is to find the best performance column in term of the highest numbers of the theoretical plates *(N)* for our research.

5.1.2 Experimental

5.1.2.1 Chemicals and materials

All chemicals and materials were the same as those experiments in Chapter 2. Atlantis HILIC packing material (3 µm diameter) and SCX (3 µm diameter Strong Cationic Exchange) material was unpacked from an Altantis HILIC (3 µm, 4.6 mm (i.d.) X 100 mm) column.

5.1.2.2 Instrumentation

Instruments were used same as those experiments in Chapter 2 and CEC in Chapter 3.

5.1.2.3 Method

There are 5 steps to make a packed HILIC column with a mid frit using photoionisation reaction as follow.

Capillary packing procedure with a mid frit

1. Preparing the γ-MAPS ready capillary
2. Creating the mid window
3. Derivatising the mid frit by Photoinitiation
4. Packing the mixed materials
5. Making the second frit

UV radiation time optimisation of making a mid frit

Three unmodified fused silica capillaries were derivatised by γ-MAPS and then dried over night. In 2009 Rassi et. al. [106] provided 2, 2-dimethoxy-2-phenylacetophenone (DMPA) to use as a photo initiator in monolithic fabrication for monolithic column. In the experiment we optimised the time of UV radiation to make a retaining frit as 5, 10, 15, 20, 25, 30 minutes of photoinitiation polymerisation time.

The best time to complete the fabrication reaction and to generate a good porosity of monolithic frit with high pressure resistance at 9000 psi is 20 minutes (n = 6). This 20 minutes photoinitiation method was optimised for making mid frit capillaries which were used for further experiment in this chapter.

Capillary Packing Procedure with a mid frit

The capillary packing process with a mid frit can be designed in the schematic charge in Figure 5.1.

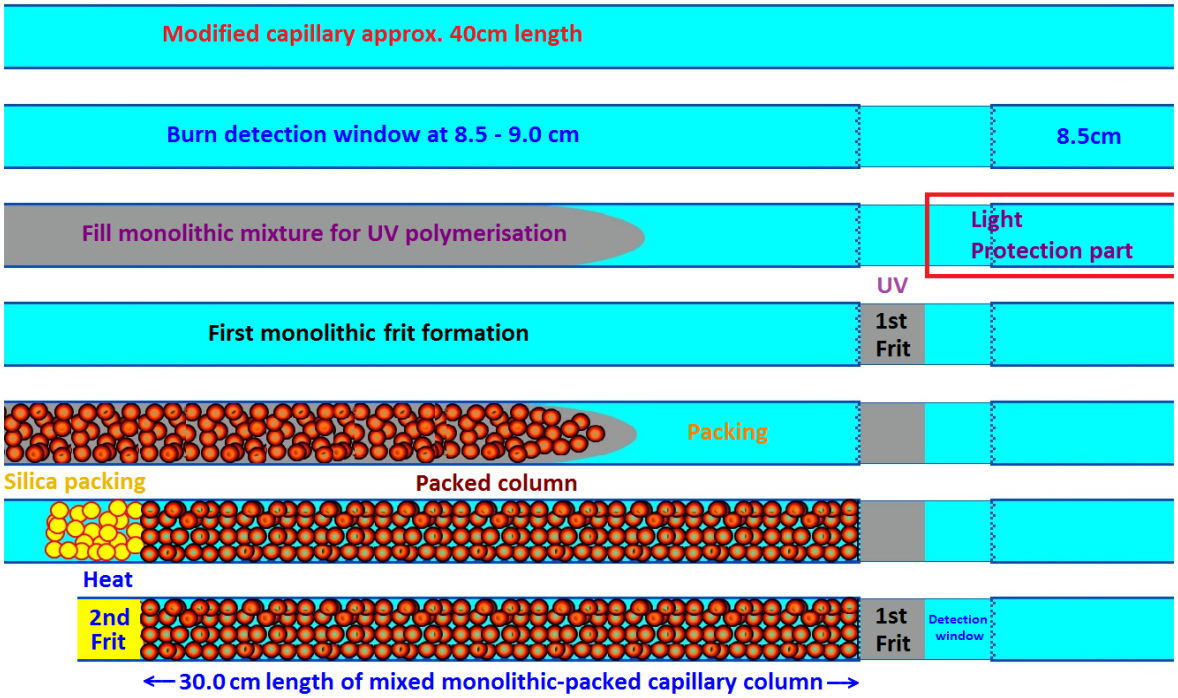


Figure 5.1 Schematic of the capillary packing procedure with a retaining mid frit by photoinitiation

5.1.2.3.1 Preparing the γ-MAPS ready capillary

Triplicate capillaries of 100 µm (i.d.) X 375 µm (o.d.) X 400 mm (length) were thermally derivatised with γ-MAPS solution at 60 ºC for 12 hours. Methanol and deionised water were used to wash the residue then nitrogen gas was passed through columns to dry overnight.

5.1.2.3.2 Creating the mid window

The γ-MAPS ready capillaries were heated 2-3 mm width at 8.5 cm length from the end of each capillary to create a clear window using a wire stripper.

5.1.2.3.3 Derivatising the mid frit by Photoinitiation

A solution of SPE-co-EDMA monolithic solution was prepared with 2% of DMPA as an initiator for UV radiation monolithic fabrication. The mixture was introduced into the capillaries using low pressure nitrogen gas. Both ends of the capillaries were closed with GC septums. Approximately half of the 2-3 mm wide detection windows which are near to the shorter end were protected from the UV reaction by opaque tubes. This restricts the area that will be polymerised. All capillaries were derivatised by using UV radiation for 5, 10, 15, 20, 25 and 30 minutes. The optimised time of reaction was successfully obtained at 20 minutes which provided excellent porosity. However, the optimised mid/retaining frit monoliths can resist the high pressure of packing at 6000 - 9000 Psi.

5.1.2.3.4 Packing the mixed materials

Atlantis HILIC packing materials were mixed with Strong Cationic Exchange (SCX) materials at 90: 10 w/w ratio using acetone HPLC grade as a solvent. The 10% slurry mixture was used to pack the mixed materials into the capillaries to the desired length (300 mm) using the packing unit.

5.1.2.3.5 Making the second frit

After finishing the packing step, a 10% silica slurry was introduced into the capillaries about 3-5 mm length to create the second frit at the end of the

capillaries. Deionised water was passed through the capillary before making the frit. The second frit was formed using the heated wire strip. Capillaries were cut to the desired length at 38.5 – 39.0 cm with 30.0 cm effective length. After finishing the column preparation from HILIC and SCX materials, it is named as the mixed-mode packed column. These mixed-mode packed HILIC/SCX columns were then washed with ACN and were used for the experiment.

The mixed-mode packed HILIC with 10% SCX columns were equilibrated with ACN: 5 mM ammonium formate pH 3.0 (90: 10) and were evaluated using micro-HPLC as the following chromatographic system, column; mixed-mode packed HILIC material with 10% SCX 100 µm (i.d.) X 375 µm (o.d.) X 30.0 cm effective length, 38.5 cm total length (n = 3), mobile phase; ACN: 5 mM ammonium formate pH 3.0 (90: 10), flow rate; 1000 nL/min, injection volume; 100 nL, detection; UV 214 nm, test compounds; toluene, acrylamide, thiourea 100 µg/mL.

After finishing the column equilibration, three HILIC test compounds were injected under the same chromatographic conditions. All stock solutions of 5 mM ammonium formate, test compound solutions and all reagents were prepared as in the same procedure as in HILIC experiment. The mobile phase was freshly prepared consisting of ACN: 5 mM ammonium formate pH 3.0 (90: 10). Finally, chromatograms from three columns were then compared in terms of the efficiency of column. After the micro-HPLC experiment, all columns were analysed by CEC using the same test compounds then compared the efficiency with the results of micro-HPLC.

5.1.3 Result and discussion

Results of the experiment including the numbers of the theoretical plates (*N*) of three sets for each test compounds (toluene, acrylamide and thiourea) and retention times are presented in Table 5.1.

Table 5.1 List of test compounds, retention times, and the numbers of the theoretical plates (*N*) comparison of mixed-mode packed HILIC (Atlantis) with 10% SCX material columns (PHX10) and sulphonic acid groups modified HILIC, poly (SPE-co-EDMA-co-2% AMPS) monolithic columns (SEA2) (n = 3)

Column	EOF (%) provider	Method	Toluene	Acrylamide	Thiourea
			N/m (Mean ± SD) [t_R (min)]	*N/m (Mean ± SD)* [t_R (min)]	*N/m (Mean ± SD)* [t_R (min)]
SEA2 (monoliths)	2.0 (AMPS)	**μ-HPLC**	6547.3 ± 40.2 [3.21 ± 0.08]	9565.1 ± 51.9 [3.91 ± 0.09]	13841.1 ± 118.4 [8.91 ± 0.22]
		CEC	40846.7 ± 1218.1 [5.19 ± 0.03]	51982.6 ± 1681.4 [5.98 ± 0.04]	62987.7 ± 1004.2 [12.07 ± 0.07]
PHX10 (pack-HILIC)	10.0 (SCX)	**μ-HPLC**	5589.9 ± 58.6 [3.33 ± 0.06]	9119.2 ± 88.6 [4.49 ± 0.11]	12281.2 ± 137.6 [16.55 ± 0.59]
		CEC	28339.2 ± 1189.3 [2.28 ± 0.07]	39118.3 ± 1382.5 [4.12 ± 0.12]	42114.5 ± 1192.4 [12.51 ± 0.43]

Example chromatograms obtained from poly sulphonic acid groups modified HILIC (SPE-co-EDMA-co-2% AMPS) monolithic columns and mixed-mode packed HILIC (Atlantis) with 10% SCX material columns are shown in Figure 5.3.

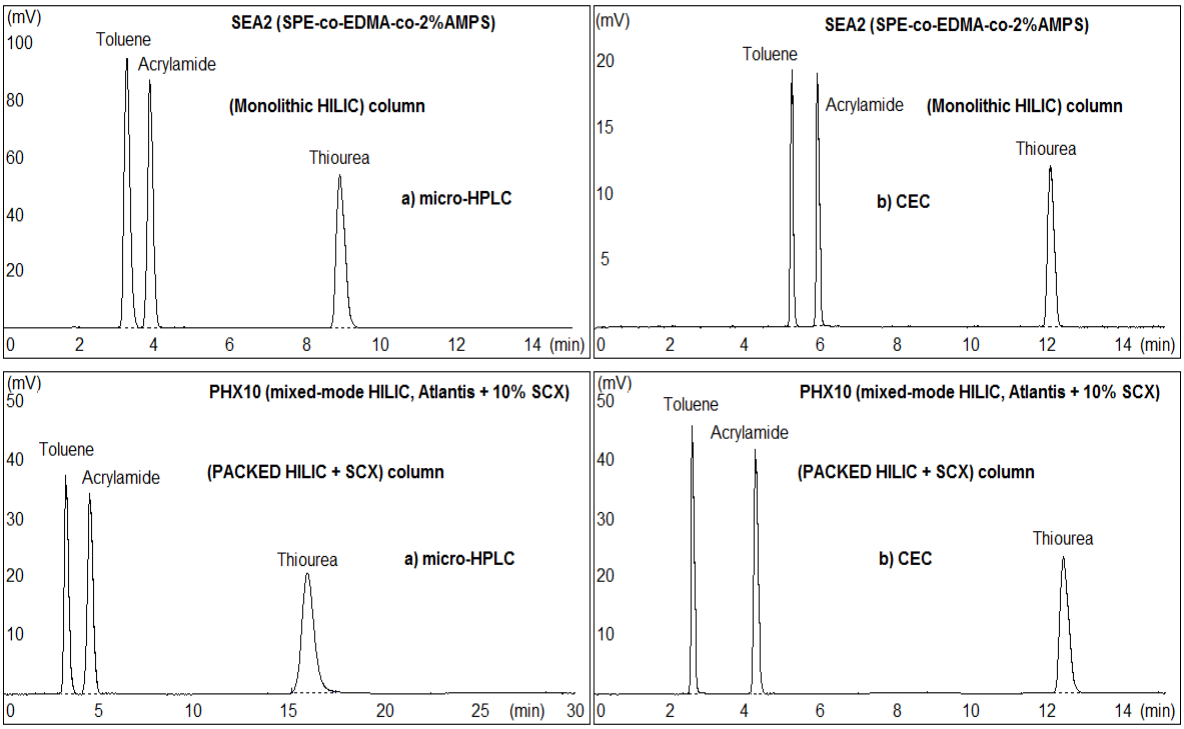


Figure 5.2 a) Micro-HPLC chromatograms of HILIC test compounds from modified HILIC, poly (SPE-co-EDMA-co-2% AMPS, SEA2) monolithic column and packed HILIC (Atlantis) with 10% SCX column and b) CEC chromatograms of HILIC test compounds using modified HILIC, poly (SPE-co-EDMA-co-2% AMPS, SEA2) monolithic column and packed HILIC (Atlantis) with 10% SCX column using mobile phase ACN: 5 mM ammonium formate pH 3.0 (90: 10)

Results of the experiment including retention times and the numbers of the theoretical plates (*N*) of each test compounds (toluene, acrylamide and thiourea) using mixed-mode HILIC (Atlantis) with 10% SCX material columns from micro-HPLC and CEC comparison with the previous best performance column, modified HILIC, poly (SPE-co-EDMA-co-2% AMPS) monolithic columns are presented in the Table 5.1 and Figure 5.2.

Having the strong cationic exchange (SCX) materials in the column increased the polarity of HILIC stationary phase and also enhanced driving force for CEC. This means the HILIC retention increases when adding the strong cationic exchange (SCX) materials into the HILIC pack column. The separation of test compounds followed the HILIC separation in order of their polarities. Toluene, the nonpolar compound eluted first, followed by neutral acrylamide, and finally thiourea, the polar compound was presented last respectively. These affected the HILIC retention of test compounds in particular the polar molecule, thiourea which was eluted last at 16.5 minutes (micro-HPLC) and 12.5 minutes (CEC). Whereas toluene, the nonpolar compound was eluted first at 3.3 minutes (micro-HPLC) and 4.1 minutes (CEC), acrylamide, the neutral compound was followed the second at 4.5 minutes (micro-HPLC) and 2.3 minutes (CEC) respectively. Having the sulphonic acid groups from the SCX material in the HILIC packing phase affected the partitioning mechanism of solutes and stationary phase, the addition of the polar moiety into the HILIC, the more polar active site in the stationary phase. Therefore, thiourea as a polar compound retained in the column longer than acrylamide and toluene respectively. In term of the numbers of the theoretical plates (*N*), the poly (SPE-co-EDMA-co-2% AMPS) monolithic column gave the better column performance for both micro-HPLC and CEC techniques than the mixed-mode pack HILIC with 10% SCX column. The numbers of the theoretical plates per metre (*N/m*) of modified HILIC poly (SPE-EDMA-co-2% AMPS) monolithic column were best on CEC at 40846.7, 51982.6 and 62987.7 for toluene, acrylamide and thiourea whereas the numbers of the numbers of the

theoretical plates per metre (*N/m*) of mixed-mode pack HILIC with 10% SCX column were also best on CEC at 28339.2, 39118.3 and 42114.5 for toluene, acrylamide and thiourea respectively.

In CEC separation (b) both modified HILIC (SPE-co-EDMA-co-2% AMPS), SEA2 column and mixed-mode pack HILIC with 10% SCX, PHX10 column successfully separated all test compounds (toluene, acrylamide and thiourea) with high efficiencies as in Table 5.1. They migrated from anode to cathode by the EOF promotion from the sulphonic acid moiety of the AMPS and by the generated charges of cationic surrounding packing materials. Then the separation happened because of the HILIC mechanism. This experiment proved the sulphonic groups from AMPS in monoliths and the charges generated from cationic beds can generate EOF to give a driving force on CEC separation for the further experiments.

In short, the performance of these packed HILIC + SCX columns were acceptable, but their efficiencies were lower with both techniques compared with the modified HILIC monolithic columns. The modified HILIC, poly (SPE-co-EDMA-co-2% AMPS) monolithic column obviously achieves the best performance in both micro-HPLC and CEC techniques. Therefore, this SPE-co-EDMA-co-2%AMPS column was considered to apply for small molecules separation in the next Chapter.

In addition, we tried a separation of the HILIC tested compounds on packed HILIC material columns without any sulphonic acid material added for CEC under the

optimised chromatographic condition. Unfortunately, there was no EOF generated from the columns to make the CEC separation occur.

Chapter 6

Applications of HILIC monolithic columns for small molecule analysis

Chapter 6. Applications of HILIC monolithic columns for small molecule analysis

In this chapter, the best performance organic monolithic columns were applied to a commercial drug analysis using both micro-HPLC and CEC techniques. As the previous chapters (Chapter 2 to Chapter 5), the organic HILIC monolithic with 2% sulphonic acid groups column showed the best column performance on both techniques. Commercial chlorpheniramine maleate tablets is a chosen model of a small molecule to ensure our columns work on both micro-HPLC and CEC analysis. In the Chapter, there are 2 experiments for a commercial chlorpheniramine maleate tablets analysis using a monolithic HILIC column and a modified HILIC monolithic column.

Chlorpheniramine maleate (CPM) is an active pharmaceutical ingredient found commonly in cough and cold formulations. The structure of which is shown in Figure 6.1.

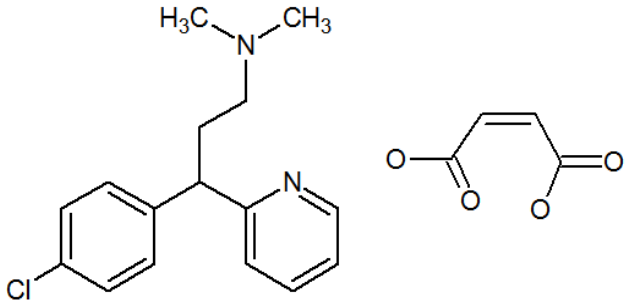


Figure 6.1 A structure of chlorpheniramine maleate

CPM is an anti-histaminic agent derived from pheniramine. It has two pK_as; for the base, chlorpheniramine; the pyridinium nitrogen has a pK_a of 3.6 and the tertiary amine has a pK_a of 9.1 [107]. The large difference in these pKa values of chlorpheniramine has made it hard to be analysed. This characteristic along with

its basic nature, makes chlorpheniramine an ideal candidate for analysis using a monolithic column. In recent research, Hadad et. al. used a monolithic column in the simultaneous analysis of CPM, caffeine, paracetamol, pseudoephedrine, aspirin and dextromethorphan [108]. CPM is often analysed in conjunction with decongestants, analgesics and caffeine due to it being formulated alongside these in pharmaceutical preparations for coughs and colds [109].

The most common method of analysis of CPM is ion-pair reversed-phase [110], [111] and recently Hadad et. al. utilised a monolith in ion-pairing mode [108] and compared it to a conventional C_{18} column. Other methods include spectrophotometry [112], nonionic micellar liquid chromatography [104], micellar electrokinetic chromatography [113], [114], liquid chromatography-mass spectrometry [115], nuclear magnetic resonance [116] and supercritical fluid chromatography [117]. The analysis of CPM is frequently determined simultaneously with other compounds such as acetaminophen (analgesic), phenylpropanolamine (sympathomimetic), paracetamol and caffeine. This is because many of the pharmaceutical formulations analysed contain more than one active pharmaceutical reagent. CPM has also been analysed in different pharmaceutical formulations for example sachets [118], suspensions [110], liquids [111], syrups [11], [120], ophthalmic solutions [121], chewing gum [122], tablets [110], [120], [123], [124], [125] and capsules [119].

Ion-pairing HPLC has been used several times for chlorpheniramine maleate (CPM) analysis. In 2001, Paciolla et. al. [111] used ion-pairing RP-HPLC, which was robust, accurate and specific for the simultaneous assay of pseudoephedrine,

doxylamine, dextromethorphan, chlorpheniramine, diphenhydramine, benzoic acid and butyl paraben in Tylenol liquid and suspension liquid products. Upon studying the effects of pH, chain length of the ion-pairing agent and ionic strength on separation, it was found that with increasing alkyl chain length of the ion-pairing agent and decreasing ionic strength lead to higher retention of the amines. CPM was very sensitive to these changes, this was because CPM has two nitrogens, which are two different pK_as and are ionisable. The method was precise and reproducible for the analysis of CPM.

Vignaduzzo et. al. [120], reported shorter retention time of chlorpheniramine, around 6 minutes, compared to 7.2 minutes achieved in this study. They successfully analysed commercial tablets and syrups containing a mixture of paracetamol, chlorpheniramine, pseudoephedrine and bromhexine. Thomas et. al. [118] showed that the using mixed ion pair liquid chromatography to assay ascorbic acid, caffeine, chlorpheniramine maleate, dextromethorphan HBr monohydrate and paracetamol in Frenadol sachets. They could achieve shorter retention times, as chlorpheniramine eluted at 2.4 minutes and maleic acid at around 9 minutes. They also found a decrease in the peak tailing using a high content, 125 mM, of tetrabutylammonium hydrogen sulphate (TBA) and an end-capped column which reduced the binding of the basic compound to the silica backbone.

Another ion-pairing HPLC was used for Anfenwanan Dispersible Tablets and was carried out by Qi et. al. [124]. The tablets contained 250 mg of acetaminophen, 15 mg of caffeine and 2 mg of chlorpheniramine maleate. CPM eluted at 7.2 minutes

and was well resolved compared to caffeine and acetaminophen. Since they used a conventional 4.6 mm column we calculated that the sensitivity increase in our method by using micro-HPLC was 30 to 50 times for limit of quantitation (LOQ) and limit of detection (LOD) respectively.

More recently, Hadad et. al. [108] compared the results of monolithic and conventional C_{18} columns in the analysis of CPM and other compounds in tablet formulations by ion-pair reversed-phase HPLC. The monolithic column showed shorter analysis times, 7 minutes in the monolithic and 18 minutes C_{18} column. The monolithic column had higher precision and accuracy with higher flow rates when compared to the conventional packed column. Upon calculation of CPM LOD and LOQ we found that the HILIC column presented here was 3 times more sensitive, in line with its smaller diameter.

The aim of this experiment is to develop and validate a method for the analysis of CPM from ALLERcalm tablets using micro-HPLC in the HILIC mode. As to date there is no report of the analysis of CPM using this method, which should achieve higher sensitivities, better peak shapes and reproducibility when compared to conventional reversed-phase (RP) and ion-pairing HPLC.

In the first experiment in this Chapter, the application of a commercial CPM tablet analysis was performed to ensure our organic monolithic HILIC, poly (SPE-co-EDMA) column successfully works for a micro-HPLC technique. Then the modified organic monolithic HILIC, poly (SPE-co-EDMA) with 2% AMPS column (the best

column) will be used for a different commercial CPM analysis on both micro-HPLC and CEC techniques in the next experiment.

6.1.1 Development and validation of the analysis of chlorpheniramine maleate from ALLERcalm tablets by micro-HPLC in the HILIC mode

6.1.1.1 Introduction

The aim of this experiment is to develop and validate a method for the analysis of CPM from ALLERcalm tablets using micro-HPLC in HILIC mode. As to date there is no report of the analysis of CPM using this method which should achieve higher sensitivities, better peak shapes and reproducibility when compared to conventional reversed-phase (RP) and ion-pairing HPLC.

6.1.1.2 Experimental

6.1.1.2.1 Chemicals and materials

SPE and EDMA were purchased from Aldrich Chemicals (Steinheim, Germany). Chlorpheniramine maleate was purchased from ICN Biomedicals; Aurora, Ohio. The ALLERcalm tablets, containing 4 mg of chlorpheniramine maleate was obtained from the manufacturer Actavis; Barnstaple, UK.

The organic solvent, acetonitrile of HPLC gradient grade, was purchased from Fischer Scientific; Leicestershire, UK. Deionised water was produced using a Millipore System (part no. SYNSV000). Ammonium formate was purchased from VWR International; Leicestershire, UK.

6.1.1.2.2 Instrumentation

The micro-HPLC system consists of the same as those experiments in Chapter 2.

6.1.1.2.3 Software

The flow rate and pressure were monitored and controlled using DiNa KYA programme. The data was acquired using Clarity, DataApex (version 5.0.1.461).

6.1.1.2.4 Chromatographic conditions

Micro-HPLC experiments were carried out at a flow rate of 1000 nL/min and detection was at 220 nm with an injection volume of 100 nL. The column used was a SPE-co-EDMA monolith (300 mm x 100 µm (i.d.) x 375 µm (o.d.)). The aqueous component of the mobile phase consisted of 5 mM ammonium formate buffer in deionised water, adjusted to pH 3.0 with 0.1 % formic acid. The mobile phase consisted of ACN: Buffer (90: 10 % v/v), which was sonicated prior to column equilibration. Chromatography was performed at ambient conditions.

6.1.1.2.5 Method

Organic HILIC (poly SPE-co-EDMA) monolithic columns were prepared same as the method in Chapter 4 (n = 3). Then the columns were used for commercial ALLERcalm tablets analysis using micro-HPLC instrument with optimised chromatographic conditions and in-house protocol.

6.1.1.2.6 Column preparation

The column was prepared by the optimised protocol as described in Chapter 2 to generate a poly N, N-dimethyl-N-(2-methacryloyloxyethyl)-N-(3-sulphopropyl) ammonium betaine –co-ethylene glycol dimethacrylate (SPE-co-EDMA) monolith. An unmodified fused-silica capillary was pre-treated with 3-(trimethoxysilyl) propyl methacrylate (γ-MAPS). It was then fabricated at 60 °C for 12 hours after filling with the polymerisation mixture consisting of a monomeric mixture (43: 57 % w/w SPE: EDMA), methanol as porogenic solvent and AIBN initiator at 33.25: 66.75: 1.00 % w/w.

6.1.1.2.7 Sample and standard preparations

6.1.1.2.7.1 Preparation of standard solutions

A stock solution of chlorpheniramine maleate was prepared at 5 mg/mL in ACN: Buffer (90: 10 % v/v). Analytical solutions were prepared from the stock solution by appropriate dilution with ACN and the buffer to achieve the same proportion of the mobile phase. Prior to use all solutions were sonicated for 10 minutes.

6.1.1.2.7.2 Preparation of sample solutions

Twenty tablets were weighed exactly and ground to a fine powder. The average weight of the tablets was calculated and 139.170 mg, 139.376 mg, 139.252 mg (equivalent to 4 mg of chlorpheniramine maleate) were weighed, respectively. The weighed amount was dissolved in 6 mL of the mobile phase and sonicated for 20

minutes. It was then diluted to 10 mL with the mobile phase after sonication followed by centrifugation for 10 minutes at 4400 rpm.

6.1.1.3 Results and discussion

6.1.1.3.1 Method development

During the optimisation process, two pH values (3.0 and 5.8), and different percentage composition of acetonitrile: buffer (85: 15 % v/v to 95: 5 % v/v), different detection wavelengths (214 nm, 220 nm and 260 nm) and flow rate (800 nL/min – 1500 nL/min) were tested. The first experiments showed that there was no separation between chlorpheniramine and maleic acid. As a result the pH was changed from 5.8 to 3.0 to increase the percentage ionisation and polarity of chlorpheniramine and maleic acid.

After separation of the two compounds was achieved, the percentage composition of the mobile phase was then investigated to acquire the best peak shape. At first a composition of 90: 10 % v/v resulted in a good separation of chlorpheniramine and maleic acid. Then a composition of 95: 5 % v/v was tested to see if the retention time between the two peaks could be shortened, but it was found that both peaks co-eluted with fronting being observed for maleic acid.

The detection wavelength was then changed from 214 nm to 220 nm then to 260 nm, in order to determine the optimum absorbance of chlorpheniramine. It was found that there was higher absorbance at 220 nm compared to 214 and 260 nm.

The flow rate was then tested in order to shorten the analysis time. Various flow rates were tried; 800 nL/min, 1000 nL/min, 1200 nL/min and 1500 nL/min. Chlorpheniramine eluted faster from 15 minutes (800 nL/min) to 7 minutes (1500 nL/min).

Upon investigation of these parameters, it was found that a chromatographic separation was successfully achieved by a mixture of mobile phase of ACN and buffer (5 mM ammonium formate pH 3.0 in deionised water) at a ratio of 90: 10 % v/v, and a flow rate of 1000 nL/min with detection at 220 nm (Figure 6.2).

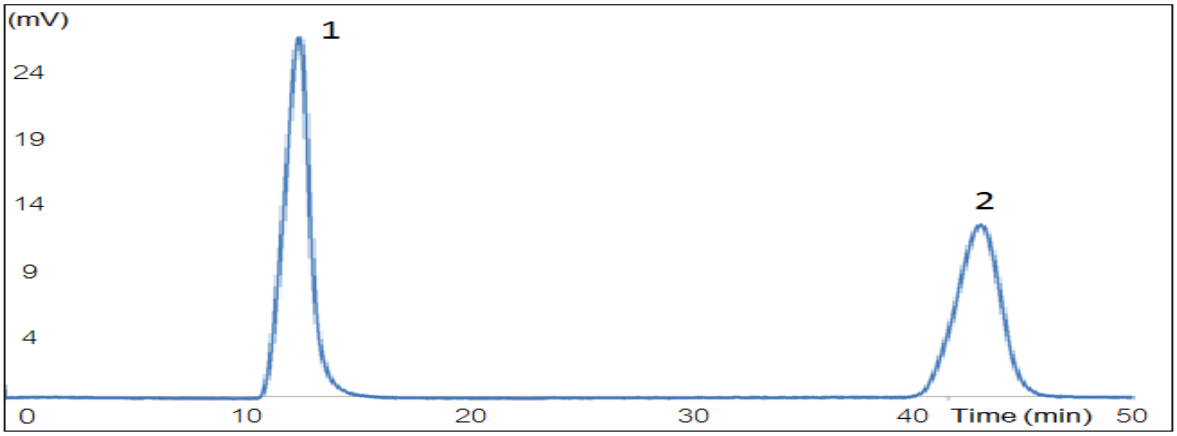


Figure 6.2 A typical micro-HPLC chromatogram of chlorpheniramine maleate standard 500 µg/mL at 1000 nL/min flow rate; 1 = chlorpheniramine, 2 = maleic acid under the optimised chromatographic conditions

To optimise the separation and shorten the time of CPM analysis, gradient elution was developed using the chromatographic conditions as; column; SPE-co-EDMA monolith (100 µm (i.d.) X 375 µm (o.d.) X 30.0 cm length), detection; 220 nm, flow

rate; 1000 nL/min, injection; 100 nL, mobile phase; (A) ACN: (B) 5 mM ammonium formate pH 3.0 in gradient mode as detailed in Table 6.1.

Table 6.1 A gradient mode scheme of standard CPM analysis for HILIC, poly (SPE-co-EDMA) monolithic column

Minute	%A	%B	Profile
0	80	20	Linear
0 - 2.0	80 - 75	20 - 25	Linear
2.1 - 5.0	75	25	Linear
5.1	80	20	Step

After the development of a gradient elution method for CPM analysis, the chromatogram shown in Figure 6.3 was obtained with chlorpheniramine and maleic acid eluting faster at 2.18 minutes and 4.38 minutes respectively.

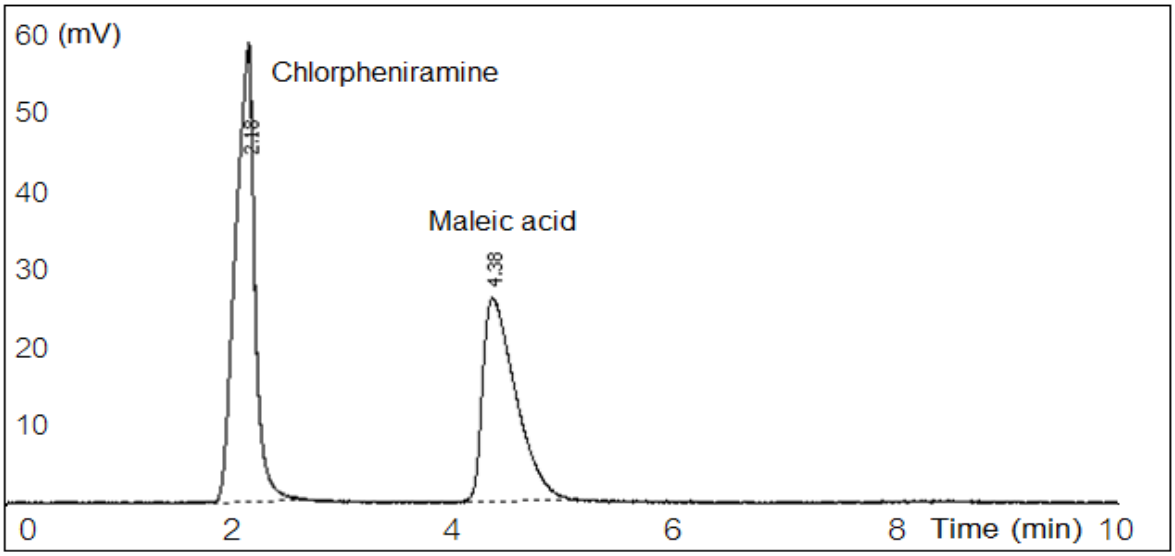


Figure 6.3 A gradient micro-HPLC chromatogram of chlorpheniramine and maleic acid from HILIC, poly (SPE-co-EDMA) monolithic column

6.1.1.3.2 Method validation

6.1.1.3.2.1 Linearity

The linearity was evaluated using CPM standards at a concentration range of 100 – 1000 µg/mL. Six injections at 100 nL and a calibration curve was plotted with CPM concentrations versus peak area (Figure 6.4).

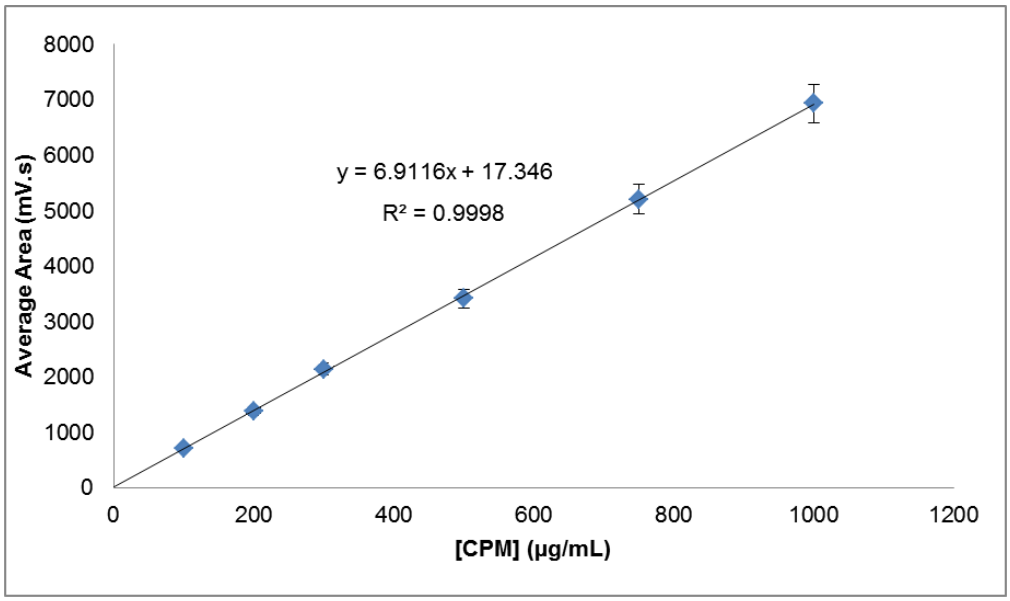


Figure 6.4 Calibration curve for standard CPM at a concentration range 100 – 1000 µg/mL using HILIC, poly (SPE-co-EDMA) monolithic column for micro-HPLC

6.1.1.3.2.2 Precision

The precision of the method was validated in terms of intra-day and inter-day percentage of relative standard deviation (%RSD) by analysing the standard CPM solution (Table 6.2).

Table 6.2 A comparison of precision in term of percentage of relative standard deviations of CPM standard (n = 6)

Standard	Percent of relative standard deviation of CPM (%RSD)	
	Intra-Day	Inter-Day
CPM	1.94	2.60

The obtained % RSDs from the Intra-Day, an analysis within the same day and from the Inter-Day, an analysis between different days are less than 3% indicating that the method is reproducible.

6.1.1.3.2.3 Sensitivity in term of detection and quantitation limits

There are several means to calculate sensitivity of the method. The guidelines for limit of detection (LOD) and limit of quantitation (LOQ) provided by ICH were displayed [126]. Using this method is based on the standard deviation of the response and the slope for determining LOD and LOQ as equations below.

The limit of detection (LOD) and limit of quantitation (LOQ) may be expressed as:

$$LOD = 3.3\sigma/S \qquad \text{Equation 16}$$

and

$$LOQ = 10\sigma/S \qquad \text{Equation 17}$$

Where σ is the standard deviation of the response (peak area), and S is the slope of the calibration curve.

Our findings of LOD and LOQ of CPM using those equations above compare with previous publications are displayed in Table 6.3.

Table 6.3 A comparison of sensitivity in term of LOD and LOQ to other published values (n = 6)

CPM	Reference	Sensitivity comparison in term of	
		LOD (pg/injection)	LOQ (pg/injection)
Found	-	276.4	837.6
Previous Analyses	Suntornsuk, et.al. [114]	852.0	2836.0
	Qi, et.al. [124]	15600.0	22400.0

When carrying out analyses on a micro scale, the sensitivity of the analysis is greatly increased. Although micro-HPLC is not conventionally used, it has many advantages over conventional HPLC such as; less mobile phase is used, which significantly reduces the cost of the purchase and disposal of the solvents which in turn reduces the amount of toxic solvents, reducing the environmental impact. The sensitivity is higher compared to conventional HPLC because of the small internal diameter of the column (500 µm – 1 mm), therefore reducing the band broadening effect during analysis [127].

6.1.1.3.2.4 Accuracy

Accuracy was determined by percentage recovery of CPM from ALLERcalm tablets. This was tested for 2 batches of tablets, with a theoretical concentration of 400 µg/mL chlorpheniramine maleate. The average percentage recovery for batch 1 and 2 were 77.95 % and 96.41 %, respectively. According to BP 2014 [128], the content limit should be found in between 92.5 % - 107.5 %. Batch 1 had failed to meet this specification, but Batch 2 has passed the BP specification (Table 6.4).

Table 6.4 Recovery data for ALLERcalm tablets

Analysis	Tablet No.	Amount of CPM in ALLERcalm tablets		
		Labelled (mg)	Found (mg)[a]	% Recovery (mg)[a]
Batch 1	1	4.00	3.21 ± 2.62	80.31 ± 2.62
	2	4.00	3.07 ± 2.62	76.72 ± 2.62
	3	4.00	3.07 ± 2.62	76.82 ± 2.62
Batch 2	1	4.00	3.78 ± 1.84	94.46 ± 1.84
	2	4.00	3.87 ± 1.84	96.86 ± 1.84
	3	4.00	3.92 ± 1.84	97.93 ± 1.84

[a]Mean ± %RSD; n = 6

6.1.1.4 Conclusion

Our developed HILIC micro-HPLC method has been shown to be highly sensitive compared to ion-pairing reversed-phase HPLC. Also the method for determination of CPM in ALLERcalm tablets has good linearity, precision, accuracy and reproducibility. The method can be directly applied for the quality control of ALLERcalm tablets in laboratories.

As mentioned in the experiment section 4.1 in Chapter 4, there is not sufficient EOF value on organic HILIC, poly (SPE-co-EDMA) monoliths for the driving force on CEC technique. Thus the modified HILIC with 2% AMPS (SPE-co-EDMA-co-2% AMPS) monolithic column was successfully developed and exhibited the best column performance. The next experiment was set to apply this best modified monolithic HILIC column to analyse chlorpheniramine in a commercial chlorpheniramine maleate tablet (Piriton) using both micro-HPLC and CEC techniques as follow.

6.2 Analysis of chlorpheniramine in Piriton tablets by modified HILIC, poly (SPE-co-EDMA) with 2% AMPS monolithic column using micro-HPLC and CEC techniques

6.2.1 Introduction

The previous experiment involving the analysis of CPM involving a neutral HILIC, poly (SPE-co-EDMA) monolithic column was carried out in the experiment on section 6.1. In this experiment, CPM was investigated using both micro-HPLC and CEC techniques under the optimised chromatographic conditions.

In addition to the monolithic column in this study, analysis will be exhibited using micro-HPLC and CEC. In micro-HPLC, the principles remain the same as in high-performance liquid chromatography (HPLC). While the mobile phase is pumped through a column at a constant flow rate, the sample is loaded onto the column via an injection port. The analytes from the sample are separated by their difference in polarity and subsequent interaction between the stationary phase and the mobile phase. Each analyte will elute from the column at a different time hence will have different retention times when they reach the detector. The miniaturisation of HPLC in micro-HPLC results in low sample consumption, and thus micro-HPLC is useful when sample availability is low. The low solvent consumption offers cost-effectiveness in solvent purchase and disposal, and reduces environmental damage [129]. In CEC, the separation depends on the combination of HPLC and CE principles as mentioned in Chapter 1. With the normal polarity of CEC, analytes will migrate from anode to cathode by EOF generating from polar

moieties of AMPS in the modified HILIC monoliths. Then the separation occurs because of HILIC mechanism.

The chromatography carried out in these series of experiments was performed in the Hydrophilic Interaction Liquid Chromatography (HILIC) mode, a term first mentioned in work by Alpert [95] in 1990. The HILIC mode uses a highly polar stationary phase with a non-polar mobile phase containing more than 50% organic solvent [130]. Typically, a solvent such as acetonitrile (ACN) is used with a low percentage of an aqueous buffer [131]. Although HILIC mode was initially intended for the analysis of polar molecules such as carbohydrates [132], peptides [133] and nucleic acids [134], it has become increasingly popular in pharmaceutical analysis [135]. This is because of its high suitability for the analysis of basic and polar compounds [136] since many drugs are of this nature. The mechanisms of separation in HILIC mode will be discussed in further detail in the discussion.

The aim of this work is to improve separation of chlorpheniramine from maleate in CPM using a charged monolithic column and compare with the analysis carried out by Experiment 6.1 which involved a neutral monolithic column. It is also intended that the method developed in this study would offer a sensitive, fast and reproducible method of analysis that could be used in low-cost quality control of CPM in a lab environment. The column used was a column made from (N,N-dimethyl-N-(2-methacryloyloxyethyl)-N-(3-sulphopropyl) ammonium betaine (SPE)–co-ethylene dimethacrylate (EDMA), HILIC poly (SPE-co-EDMA) with 2% 2-acrylamido-2-methylpropane sulphonic acid (AMPS) monolith stationary phase. The polar moiety from sulphonic acid groups in AMPS makes a highly hydrophilic

stationary phase and also generates an EOF driving force for CEC analysis. This column will be referred to as the poly (SPE-co-EDMA-co-2% AMPS) monolithic column. The column was prepared in situ.

In this work, the best HILIC, poly (SPE-co-EDMA) modified with 2% AMPS monolithic column was used in the quantitative and qualitative analysis of chlorpheniramine maleate, a small basic molecule, from Piriton tablets.

6.2.2 Experimental

6.2.2.1 Chemicals and materials

N,N-dimethyl-N-(2-methacryloyloxyethyl)-N-(3-sulphopropyl) ammonium betaine (SPE), ethylene dimethacrylate (EDMA), 3-(trimethoxysilyl) propyl methacrylate (γ-MAPS) and (2-acrylamido-2-methyl-1-propanesulphonic acid (AMPS) were purchased from Aldrich Chemicals (Steinheim, Germany). Ammonium formate and N-cyclohexyl-3-aminopropanesulphonic acid (CAPS) were purchased from Sigma, and CPM was obtained from ICN Biomedicals inc. A Millipore system (serial #SYNSV000) was used to prepare deionised water, and HPLC-grade ACN and methanol were sourced from Fischer Scientific (Leicestershire, UK). Chlorpheniramine maleate tablets containing 4 mg of chlorpheniramine (CPM) were purchased from GSK branded as Piriton, batch number 188443 (expiry date 03/2015). The fused silica capillaries were obtained from CM Scientific (Silsden, UK, Part #TSP100375).

6.2.2.2 Instrumentation

Micro-HPLC experiments were carried out using a DiNa gradient pump, with a four port valco injection valve and a 100 nL internal loop. The UV detector was an Applied Biosystems 783 programmable absorbance detector with a capillary cell. Data acquisition and processing was carried out using DataApex Clarity chromatography station. The oven used for the thermal polymerisation process was obtained from Genlab (Cheshire, UK). The microscope used during column preparation to check for presence of air bubbles inside the capillary was purchased from Jencons-Pls (model #00035160). The thermal stripper was sourced from Innova Tech (Stevenage, UK). The centrifuge used was a Heraeus.Megafuge. 8 centrifuge (Thermo, Waltham, MA, USA). The pH meter used during buffer pH adjustment was a Mettler Delta 320 meter, purchased from Mettler Toledo (Halstead, UK). Sonication was carried out using a XUBA3 ultrasonic bath, sourced from Grant Instruments (Cambridgeshire, UK).

6.2.2.3 Method

Three modified monolithic HILIC columns (SPE-co-EDMA-co-2% AMPS) were prepared using the same method in as described in Experiment 4.1 (section 4.1.2.3) in Chapter 4. Then the columns were used for commercial Piriton tablets analysis using micro-HPLC and CEC instruments.

6.2.2.3.1 Column preparation

The capillaries were treated with the bifunctional reagent, γ-MAPS, to covalently attach the polymer to the inner capillary wall prior to polymerisation, according to a previously used method [137]. The polymerisation mixture containing 43% w/w of the functional monomer SPE, 2% AMPS monomer, 57% w/w of the cross linker EDMA, the initiator AIBN (~ 1% w/w with respect to the monomer), 0.6675 g of the porogenic solvent methanol were weighed into a 2 ml vial after which the mixture was ultrasonicated to obtain a homogenous solution. The polymerisation mixture was sonicated and bubbled with nitrogen gas for approximately 5 minutes to remove dissolved gases such as oxygen which would interfere with monolith formation within the capillary. The polymerisation mixture was then introduced into the pre-treated capillary, and a microscope was used to confirm that this had been done successfully and that there were no air bubbles. Both ends of the capillary were immediately sealed off with GC septa. The filled capillary was placed in the oven for 12 hours at 60 ºC. Once polymerisation was complete, the capillary was connected to a packing pump and flushed with ACN: deinoinised water (50: 50 % v/v) to remove any unreacted materials and porogenic solvent. A thermal striper

was then used to pyrolyse the monolith to create a 2-3 mm detection window at a distance of approximately 8.5 cm from one end of the column. It was then cut to the desired length (30.0 cm effective length and 38.5 total length) from the detection window to the beginning of the column.

6.2.2.3.2 Preparation of solutions

6.2.2.3.2.1 Preparation of buffers

For the pH 3 buffer, 157.0 mg of ammonium formate was weighed out and made up to 500 mL in a volumetric flask with distilled water. The resulting 5 mM solution was sonicated and adjusted to pH 3.0 using 0.1% v/v formic acid. For the pH 11 buffer, 222.0 mg of CAPS was weighed out and made up to 200 mL with distilled water. The resulting 5 mM solution was sonicated and adjusted to pH 11.0 using 1% w/v NaOH. The buffers were prepared on a weekly basis during the course of the experiments and refrigerated to prevent microbial growth [138] which could risk column damage.

6.2.2.3.2.2 Preparation of reference standards

Stock solutions of the reference standards were prepared by weighing 20.0 mg of toluene, acrylamide and thiourea in separate volumetric flasks and making up to 10 mL using HPLC grade ACN. The stock solutions were then further diluted by aliquoting 100 µl of each stock solution into a single volumetric flask, and then 600 µl of ACN and 100 µl of ammonium formate pH 3.0 buffer were added.

6.2.2.3.2.3 Preparation of mobile phases

Mobile phase 1 (90: 10 %v/v) and mobile phase 2 (95: 5 %v/v) of ACN: 5 mM ammonium formate buffer pH 3.0 were prepared from the ACN (HPLC grade) and 5 mM ammonium formate buffer pH 3.0 solution. Additionally, mobile phase 3 (95: 5 %v/v) of ACN: 5 mM CAPS buffer pH 11.0 was also freshly made correspondingly.

6.2.2.3.2.4 Preparation of CPM stock solution

A stock solution of CPM was prepared at a concentration of 5.0 mg/mL, where 50.0 mg of CPM was weighed and dissolved in 10 mL of ACN. This stock solution was further diluted where 0.05 mL of the stock solution was aliquoted and diluted to 1 mL with 0.9 mL of ACN and 0.05 mL of the appropriate buffer (pH 11.0 or pH 3.0 as required).

6.2.2.3.2.5 Preparation of Piriton tablet sample solutions

In the first extraction method attempted, 20 Piriton tablets were weighed according to the British Pharmacopoeia assay [139], and then ground to a fine powder using a pestle and mortar. The average weight of one CPM tablet was calculated and the according weight of powder was aliquoted. The weighed amount was dissolved in 6 ml of ACN, and sonicated for 20 minutes and then centrifuged for 10 minutes. The supernatant was transferred into a separate vial to be made up to the 10 mL volume with appropriate proportion of ACN and the buffer to achieve the desire mobile phase before injecting into the column.

In the second extraction method attempted, one tablet was placed in an Eppendorf tube and comminuted into powder using a tissue-lyser. The resultant powder was dissolved in 6 mL of ACN, and sonicated for 20 minutes and then centrifuged for 10 minutes. The supernatant was transferred into a separate vial to be made to 10 mL volume with ACN and the buffer getting desired the same proportion as the mobile phase.

6.2.2.3.3 Chromatographic conditions

The experiments were performed at room temperature at a flow rate of 1000 nL/min with UV detection at 220 nm. Both newly prepared mobile phases were degassed by sonication prior to use in the column equilibration. All sample solutions were sonicated prior to injection. The column used was the modified HILIC poly (SPE-co-EDMA-co-2% AMPS). The dimension of the column is 100 µm internal diameter (i.d.), 375 µm outer diameter (o.d.) with an effective length of 30.0 cm. In addition, each analysis was carried out 6 times and its average values were used.

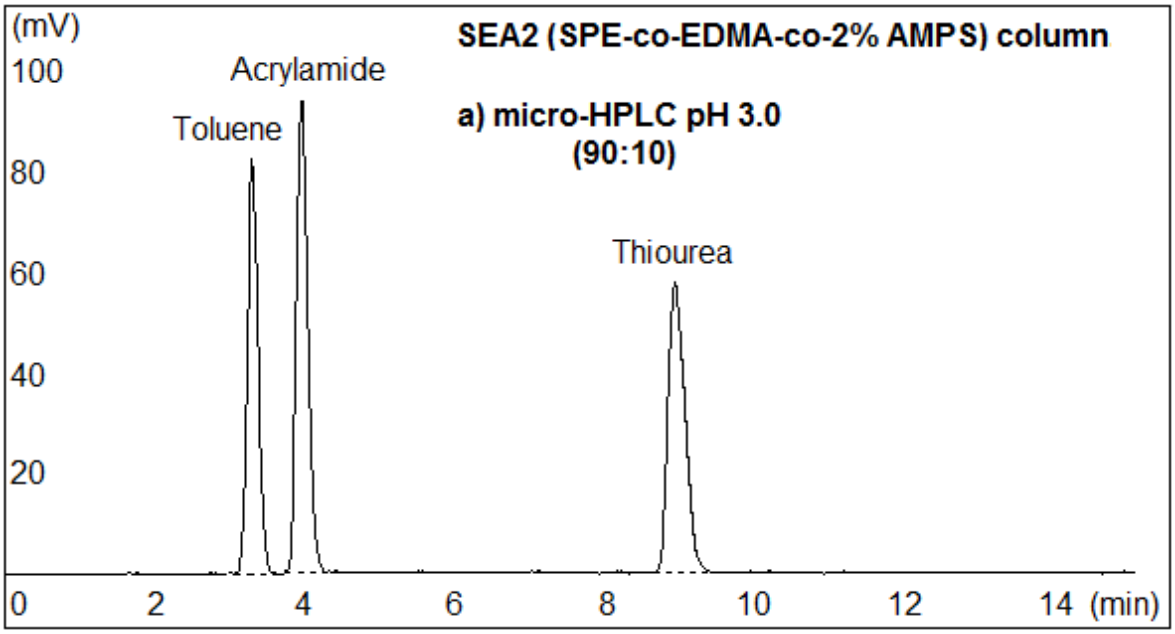
6.2.3 Results and discussion

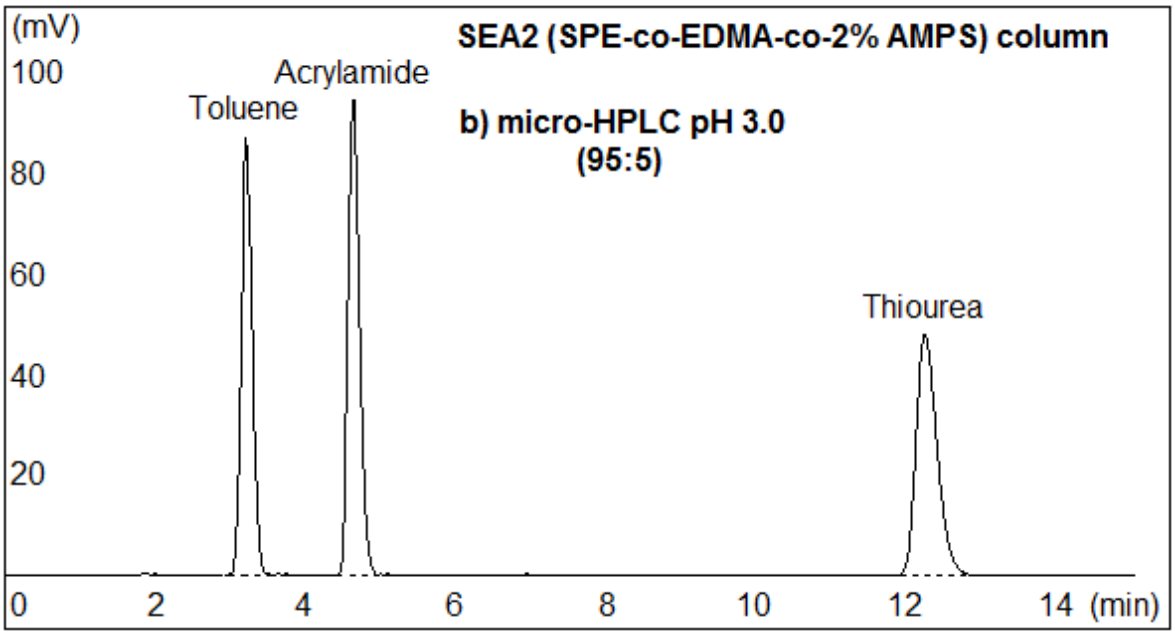
6.2.3.1 Method development

6.2.3.1.1 Proving the columns' HILIC properties and the HILIC mechanism of separation

The theory of HILIC separation mechanism is that there is a pseudo-stationary, with a partially immobilised water-rich layer surrounding the hydrophilic stationary phase [140]. There is partitioning of the analyte between the water-rich pseudostationary phase and the acetonitrile-rich mobile phase [140], allowing separation from the bulk of the mobile phase. The more hydrophilic the analyte is, the more it will preferentially distribute into the water-rich layer and be retained for longer. However, it has been concluded that in reality, separation rarely occurs due to 'pure HILIC' interaction based on partitioning, instead occurring due to a complex combination of multiple interactions [141] between the analyte, stationary phase and mobile phase. These include partitioning and absorption-desorption due to electrostatic interactions, hydrogen bonding and dipole-dipole forces [140]. Firstly, it was confirmed that the column used was demonstrating HILIC-properties. This was carried out by injecting a standard mixture of toluene, acrylamide and thiourea [142]. Toluene, the most hydrophobic compound in the mixture, eluted first followed by acrylamide and thiourea, which was the most hydrophilic. This was confirmed by single injections of each of the compounds and their retention times were compared in order to verify their identity. The column demonstrated HILIC behavior, as shown in Figure 6.5 a) and Figure 6.5 b) and c) are

chromatograms of HILIC test compounds and CPM run on the same column using micro-HPLC at ACN: buffer (90: 10) and (95: 5) at pH 3.0, respectively.





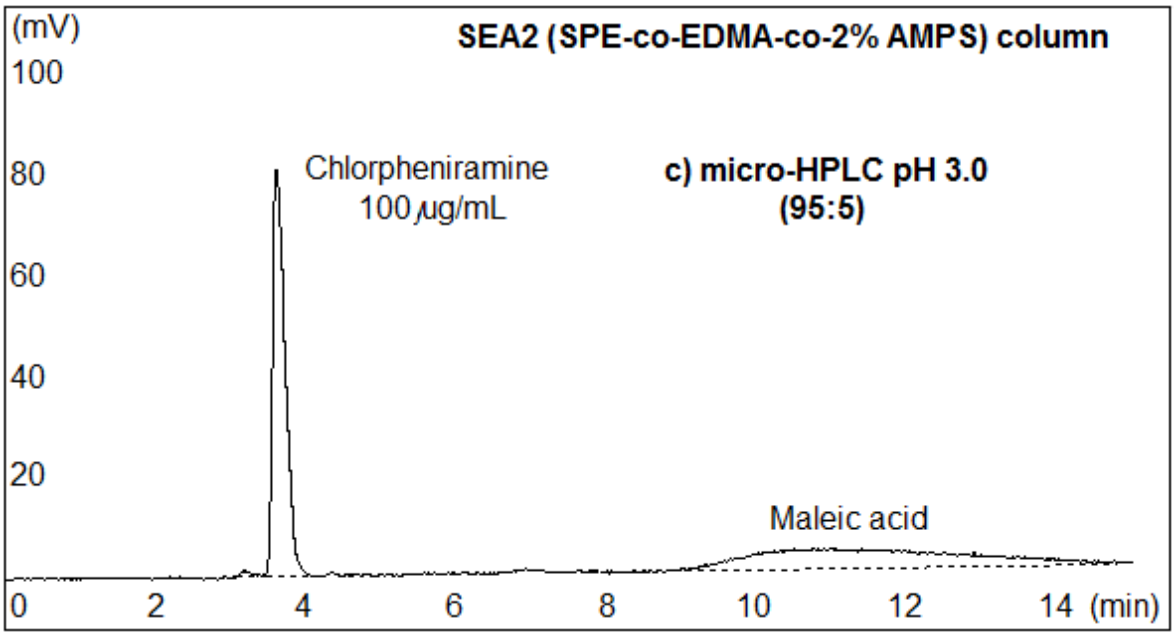


Figure 6.5 Chromatograms for the test mixture of toluene, acrylamide and thiourea and chlorpheniramine maleate in mobile phase; ACN: 5 mM ammonium formate pH 3.0 (a) 90: 10 and (b), (c) 95: 5) using the poly (SPE-co-EDMA-co-2% AMPS) monolithic column

6.2.3.1.2 Selection of a mobile phase for micro-HPLC

Using mobile phase 1, ACN: 5 mM ammonium formate pH 3.0 (90: 10) the averages retention times (t_R) of toluene, acrylamide and thiourea were at 3.16, 3.99 and 8.97 minutes respectively whereas it was 3.23 minutes for chlorpheniramine. It was found that the retention time of chlorpheniramine (t_R) was very close to that of toluene using this column, giving only 4.2 seconds difference between them. Since toluene is an un-retained compound, the co-elution meant that using this mobile phase was not suitable to analyse chlorpheniramine. Therefore optimisation of mobile phase 1 should be considered.

Upon changing the mobile phase composition from mobile phase 1 (90: 10 % v/v ACN: running buffer pH 3.0) to mobile phase 2 (95: 5 % v/v ACN: running buffer pH 3.0), a full separation between chlorpheniramine and toluene was achieved. Therefore the optimised mobile phase was at a ratio of acetonitrile/ running buffer pH 3.0 at 95: 5 % v/v which provided an average retention time (t_R) of toluene and chlorpheniramine at 3.19 and 3.59 minutes respectively. The protonated chlorpheniramine is clearly more polar than toluene at this pH and is therefore more retained. Because the AMPS component of the monolith is also ionised, electrostatic interaction will form part of the retention mechanism.

In summary, the optimised micro-HPLC chromatographic system for chlorpheniramine maleate tablet analysis using a poly (SPE-co-EDMA) with 2% AMPS monolithic column was achieved using a mobile phase of ACN and running buffer (5 mM ammonium formate pH 3.0) at a ratio of 95: 5 % v/v, and a flow rate of 1000 nL/min with detection at 220 nm (Figure 6.5 b) and c)). The analysis of chlorpheniramine in Piriton tablet by micro-HPLC was carried out using this optimised condition.

6.2.3.1.3 Selection of a buffer and pH for CEC

For the CEC experiment, ammonium formate buffer was adjusted to pH 3.0 using formic acid. The background electrolyte (BGE) for the CEC experiment was the same as the mobile phase using for micro-HPLC. The chromatographic conditions used for CEC were; column; a modified HILIC, poly (SPE-co-EDMA) monolithic column with 2% AMPS, 100 µm (i.d.) X 375 µm (o.d.) X 300 mm (n = 3), (30.0 cm

effective length, 38.5 cm total length). Background electrolyte (BGE); ACN: 5 mM ammonium formate pH 3.0 (95: 5). CEC mode; normal polarity, +30.0 kV. Injection; 50 mBar X 1000 seconds. Detection; UV 220 nm. Using these conditions, it was found that the separation of the HILIC test mixture (toluene, acrylamide and thiourea) was successfully performed.

Firstly, the characteristic of modified HILIC monoliths with sulphonic acid groups, poly (SPE-co-EDMA-co-2% AMPS) was considered at pH 3.0. At this pH both the chlorpheniramine and the sulphonic acid groups from AMPS will be ionised. AMPS is negatively charged at pH 3.0 and Chlorpheniramine is positively charged so there will be electrostatic interactions between AMPS and chlorpheniramine. However, it appears that using CEC under the conditions used in micro-HPLC to retain chlorpheniramine, results in chlorpheniramine eluting before toluene. One would have expected the protonated chlorpheniramine to be very polar and therefore interact with the stationary phase and be longer retained. Also, the charged chlorpheniramine would be expected to undergo a degree of electrostatic interaction with the AMPS component, also increasing retention. However, the fact that chlorpheniramine elutes before toluene under these conditions is probably related to the joint effect of the EOF and electrophoretic mobility resulting in a fast linear velocity through the bed.

Secondly, the ionisation state of chlorpheniramine should be established under the test pH. At pH 3.0, there is protonation of the pyridinium nitrogen and the tertiary amine on chlorpheniramine, making it double positively charged. When running a normal polarity CEC, the positively charged chlorpheniramine will migrate towards

the cathode because of its electrophoretic mobility and favorable EOF, while the separation of test compounds (toluene, acrylamide and thiourea) occur simply due to partitioning according to the HILIC mechanism. However, the migration order of chlorpheniramine and test compounds was unfavorable since toluene is an un-retained compound [143], meaning it will travel through the column with the EOF without interacting with the stationary phase. Because chlorpheniramine eluted before toluene, this confirmed that it also was not being retained. The fact that it eluted before toluene, confirms the fact that elution is a result of a combination of electrophoretic mobility and EOF.

Un-retained chlorpheniramine on this column showed the mobile phase 2 was not acceptable for chlorpheniramine analysis by CEC.

At pH 3.0, maleic acid which has pKa at 1.9 was mainly negatively charge. Thus, it was fully retained at the beginning of the column and will not migrate towards to the cathode because of the charge attraction with the anode when running normal polarity CEC.

For these reasons, the optimal pH for chlorpheniramine analysis using this modified monolithic column needed to be considered further. A study of optimal pH was then set at pH 11.0 (mobile phase 3) to achieve the retaining of chlorpheniramine and improve the analysis by CEC. The CEC chromatograms at pH 3.0 of a) HILIC test mixture and b) chlorpheniramine are shown in Figure 6.6.

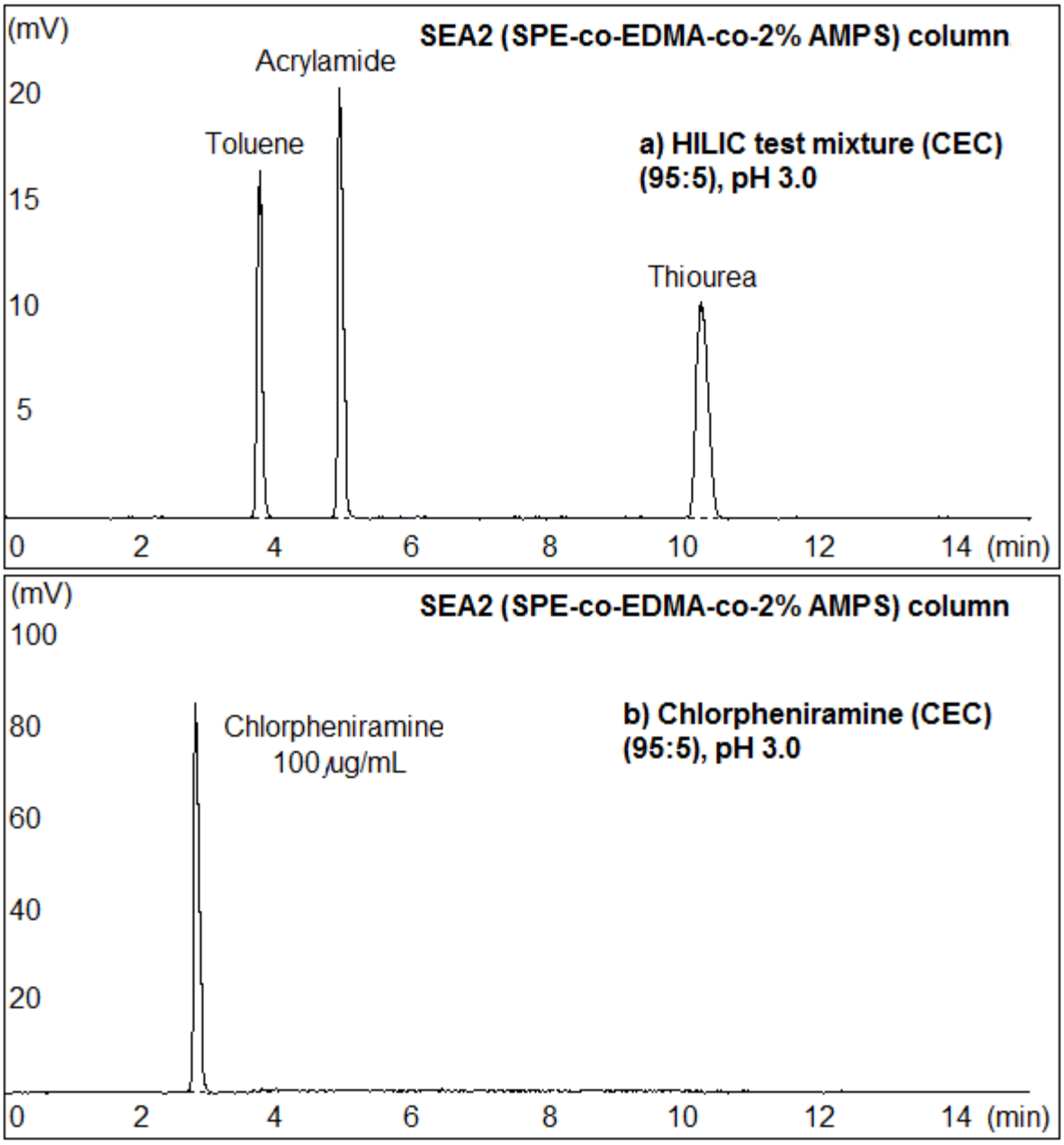


Figure 6.6 Chromatograms of a) toluene, acrylamide and thiourea and b) chlorpheniramine in background electrolyte (mobile phase 2); ACN: 5 mM ammonium formate pH 3.0 (95: 5) using poly (SPE-co-EDMA-co-2% AMPS) column

At pH 11.0 (mobile phase 3), the AMPS moiety in the modified monoliths are fully negatively charge which can generate enough EOF driving force for a normal polarity CEC. At this high pH however, chlorpheniramine is neutral since pH 11.0 is above both its pKa value of 3.6 and 9.1. Due to chlorpheniramine being neutral, any interactions with the column would have been mostly HILIC partitioning, dipole-dipole forces and hydrogen bonds. The absence of any charge on it meant there was no electrostatic interaction with the stationary phase in this column compared to pH 3.0 where it is charged. The neutral chlorpheniramine and HILIC test compounds will migrate along the column purely as a result of the EOF driving force arising from the charged sulphonic acid groups of AMPS and their mass differences employing HILIC mechanism. Unlike with the micro-HPLC experiments, this is reflected in the retention time, where chlorpheniramine is retained longer under basic conditions.

With micro-HPLC at low pH (pH 3.0), chlorpheniramine is longer retained than it is at high pH (pH 11.0). This is because at low pH with micro-HPLC, chlorpheniramine and AMPS are both ionised so also they get electronic interactions leading to longer retention.

With CEC at high pH (pH 11.0), chlorpheniramine is neutral so less likely to get much electrostatic interaction, or hydrophilic interaction because it is less polar at this pH and also the elution is a result of EOF only. More retention for chlorpheniramine with CEC at high pH compared to low pH because at pH 3.0 the elution governs by electrophoretic mobility and EOF. Therefore it is longer retained at pH 11 resulting better chlorpheniramine analysis. For this reason, mobile phase

3 (pH 11.0) was considered to perform better separation of HILIC test compounds and chlorpheniramine to ensure it will achieve good separation without co-elution with toluene or acrylamide, meaning chlorpheniramine was sufficiently being retained by the column. Again for maleic acid at pH 11, it was fully negatively charged when running normal CEC. Therefore it will not migrate towards the anode as the charge attraction.

The CEC experiment at pH 11.0 of a background electrolyte using mobile phase 3 was successfully performed. At pH 11.0, the buffer initially chosen was ammonium bicarbonate, adjusted to pH 11.0 using sodium hydroxide. However, it was found that a cloudy precipitate was formed due to salt production when using a high proportion of organic solvent (acetonitrile), and this buffer could not be used since this would have clogged the system and also the column. Thus, an organic buffer was chosen (CAPS) adjusted to pH 11.0 using sodium hydroxide, which had a buffering range of 9.7-11.1 [144]. It was found that preparation of fresh buffer was essential. When the buffer used was not prepared fresh weekly and not refrigerated, the chromatography suffered fluctuating baseline and rising backpressure, perhaps due to microbial growth on the column [138] or salt formation.

The new background electrolyte (mobile phase 3) was prepared and used for chlorpheniramine analysis experiment by a normal polarity CEC. This optimal chromatographic condition for CEC was; column; a modified HILIC, poly (SPE-co-EDMA-co-2% AMPS) monolithic column, 100 µm (i.d.) X 375 µm (o.d.) X 300 mm column (n = 3), (30.0 cm effective length, 38.5 cm total length), background

electrolyte (BGE); ACN: 5 mM CAPS buffer pH 11.0 (95: 5), CEC mode; normal polarity, +30.0 kV, injection; 50 mBar X 1000 seconds, detection; UV 220 nm.

The HILIC test mixture (toluene, acrylamide and thiourea) was successfully separated and was proved for the column's HILIC properties at pH 11. In addition, chlorpheniramine was clearly eluted after the non-polar toluene and after neutral acrylamide employing HILIC mechanism. Non-polar toluene eluted first, followed by neutral acrylamide and neutral chlorpheniramine then the most polar thiourea respectively. Chlorpheniramine eluted after acrylamide because of its higher molecular weight.

Therefore the analysis of chlorpheniramine in Piriton tablet by CEC was successfully carried out using this optimised condition at pH 11.0. The CEC chromatograms of HILIC test mixture and chlorpheniramine using this best column are shown in Figure 6.7.

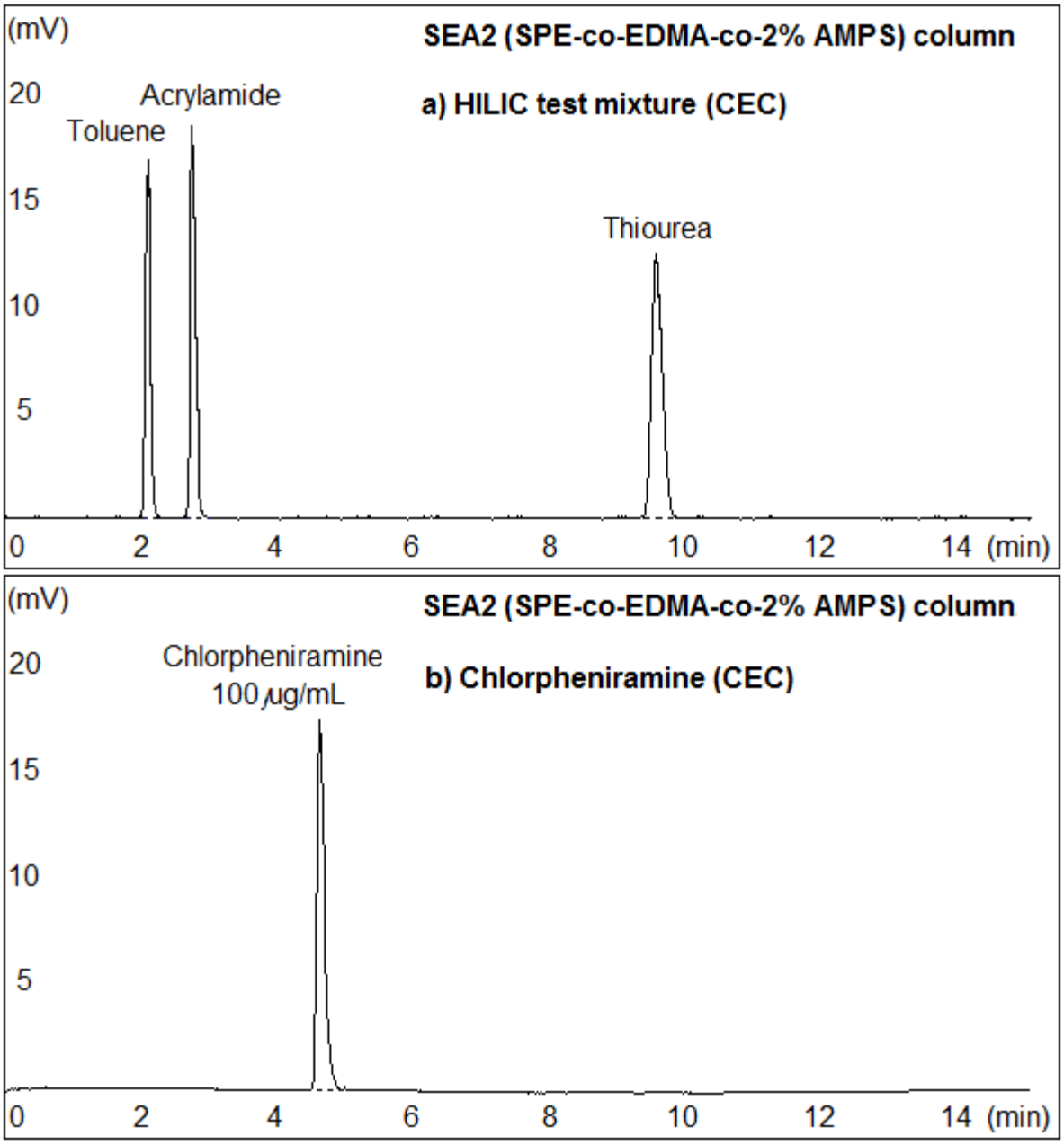


Figure 6.7 Chromatograms of a) toluene, acrylamide and thiourea and b) chlorpheniramine in background electrolyte (mobile phase 3); ACN: 5 mM CAPS buffer pH 11.0 (95: 5) using poly (SPE-co-EDMA-co-2% AMPS) column by CEC

6.2.3.2 Method validation

6.2.3.2.1 Linearity

Calibration curves for (a) micro-HPLC and b) CEC) were constructed in order to validate the linearity of the developed methods. These were done using standard solutions of chlorpheniramine maleate at a concentration range of 100-1000 µg/mL. Six injections were made at each concentration, and a six-point (100, 200, 300, 500, 750 and 1000 µg/mL of chlorpheniramine) calibration curve was plotted. Six injections were made for each concentration.

For micro-HPLC, the regression equation obtained was:

$$\text{Peak area} = 1.6725[\text{CPM (µg/mL)}] + 0.9825$$

The correlation coefficient obtained (R^2) was 0.9996.

For CEC, the regression equation was:

$$\text{Peak area} = 1.4671[\text{CPM (µg/mL)}] + 1.3574$$

The correlation coefficient obtained (R^2) was 0.9996.

From the correlation coefficients of both developed techniques, the values were higher than 0.995, which indicated a strong linear relationship between CPM concentration and peak area. Therefore the developed methods were linear within the investigated concentration range of 100-1000 µg/mL as shown in Figure 6.8.

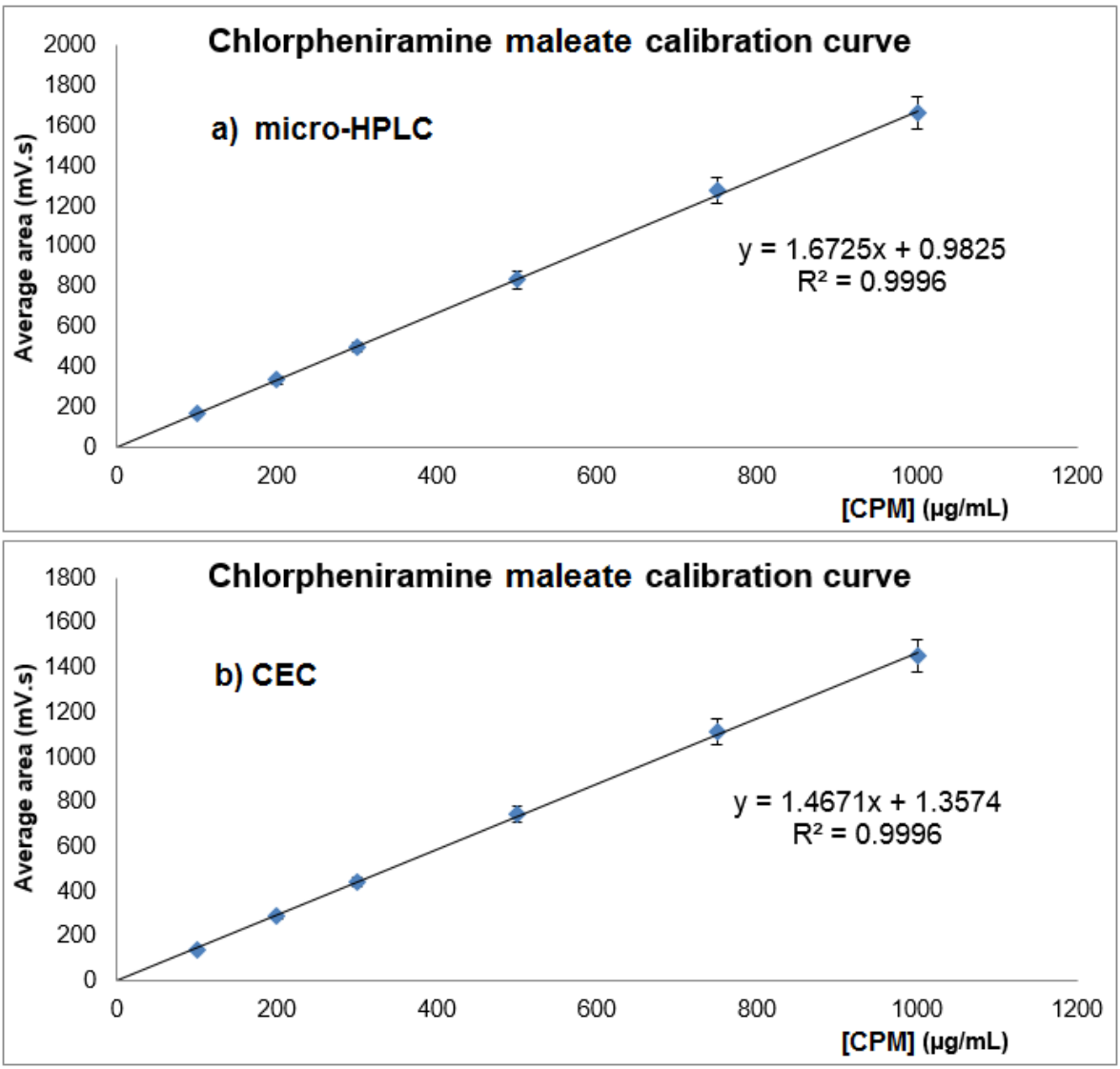


Figure 6.8 Six-point calibration curves showing linear relationship between the concentration of CPM and resulting peak area using a) micro-HPLC and b) CEC

6.2.3.2.2 Precision

The repeatability of the retention time *(t_R)* of chlorpheniramine from the standard solution was tested at a sample size of six (n = 6) for both techniques. The standard deviations obtained were less than 0.005 indicating that the developed methods had high precision, as shown in Table 6.5.

Table 6.5 The retention times and standard deviations of the average retention times of chlorpheniramine in different runs using micro-HPLC and CEC

Method	Percent of relative standard deviation of CPM (%RSD)	
	Intra-Day	Inter-Day
micro-HPLC	0.649	0.871
CEC	0.913	1.360

6.2.3.3.3 Detection and quantification limits

Limit of detection (LOD) and limit of quantification (LOQ) were calculated using the International Conference on Harmonisation (ICH) guidelines [126], where the standard deviation of the peak area and the slope were used to calculate LOD and LOQ using Equation 16 and Equation 17 in section *6.1.1.3.2.3*.

The LOD and LOQ of CPM for micro-HPLC and CEC were calculated using those equations compare with previous publications are displayed in Table 6.6.

Table 6.6 A comparison of sensitivity in term of LOD and LOQ to other published values (n = 6)

CPM	Reference	Sensitivity comparison in term of	
		LOD (pg/injection)	LOQ (pg/injection)
Method Found	micro-HPLC	298.9	905.9
	CEC	243.4	737.5
Previous Analyses	Suntornsuk, et.al.[114]	852.0	2836.0
	Qi, et.al.[124]	15600.0	22400.0

6.2.2.3.4 Accuracy

Accuracy was determined by percentage recovery of CPM from Piriton tablets using both micro-HPLC and CEC techniques. These were tested for 2 batches of tablets, with a theoretical amount of 4.0 mg chlorpheniramine maleate equivalent. Using the equations from Figure 6.8 to calculate CPM amounts for each injection (n = 6), founded CPM were shown in Table 6.7 with recovery percentages. From the results in Table 6.7, the average recovery percentages for batch 1 and 2 were 99.59 % and 99.32 % for micro-HPLC and were 97.97 % and 98.21 % for CEC, respectively. According to BP 2014 [128], the content limit should be found in between 92.5 % - 107.5 %, all batches passed the BP specification for both micro-HPLC and CEC.

Table 6.7 CPM recovery data comparison between previous analyses and our methods (micro-HPLC and CEC)

Method	Analysis	Tablet No.	Amount of CPM in ALLERcalm tablets		
			Labelled (mg)	Found ± SD (mg)	Recovery (%)[a]
micro-HPLC	Batch 1	1	4.00	3.98 ± 0.07	99.60 ± 1.85
		2	4.00	4.00 ± 0.08	100.03 ± 1.97
		3	4.00	3.97 ± 0.08	99.18 ± 1.92
	Batch 2	1	4.00	3.96 ± 0.08	98.99 ± 1.99
		2	4.00	3.97 ± 0.08	99.15 ± 2.08
		3	4.00	3.99 ± 0.09	99.83 ± 2.14
CEC	Batch 1	1	4.00	3.94 ± 0.09	98.48 ± 2.28
		2	4.00	3.89 ± 0.07	97.12 ± 1.84
		3	4.00	3.93 ± 0.07	98.24 ± 1.92
	Batch 2	1	4.00	3.91 ± 0.08	97.73 ± 2.05
		2	4.00	3.94 ± 0.08	98.52 ± 2.03
		3	4.00	3.93 ± 0.09	98.36 ± 2.29

[a]Mean ± %RSD; n = 6

6.2.4 Conclusion

Sharp peak shapes were obtained at pH 3 with a mobile phase of 95: 5 % v/v acetonitrile to 5 mM ammonium formate buffer using this modified HILIC monolithic column. Calibration curves from both micro-HPLC and CEC results were constructed using the drug standard and validated in terms of linearity, precision and detection and quantification limits. Quantifications of the drug from Piriton tablets for both micro-HPLC and CEC were successful. For micro-HPLC, a gradient analysis improved peak shapes and shortened analysis times to 3.59 minutes. For CEC, a better performance of column was performed with higher sensitivity than micro-HPLC.

Overall, a rapid and reproducible method was developed for the analysis of chlorpheniramine from the drug standard, with shorter analysis times and better column performance compared to existing work. A reproducible and fast method was developed for the analysis of chlorpheniramine using micro-HPLC and CEC on a modified HILIC monolithic with sulphonic acid groups (poly SPE-co-EDMA-co-2% AMPS) column. The column successfully demonstrated HILIC properties and quantified chlorpheniramine from the commercial chlorpheniramine maleate tablets (Piriton) with good peak shapes. The analysis time was significantly shorter compared to that achieved using the neutral HILIC, poly (SPE-co-EDMA) monolithic column in the previous experiment (section 6.1). Method validations on both micro-HPLC and CEC were successfully performed. The labelled amount of chlorpheniramine in Piriton tablet all Batches from both techniques passed the BP specification. The extraction process of CPM from Piriton could be further explored, in addition to test the effects of changing column temperature on separation of CPM. Due to the many advantages monolithic columns offer in the analysis of basic drugs, and the excellent suitability of HILIC mode for polar molecule analysis, we can expect to see them being used more widely in conjunction with each other in pharmaceutical analysis.

Chapter 7

Conclusions and future works

Chapter 7. Conclusions and future works

7.1 Conclusions

Capillary chromatography has been developed and used for small molecule analysis for decades because of it's benefit of more sensitivity and efficiency. There has been much research into the development of new stationary phases for small molecule analysis. Manufactured packed capillary columns have poor reliability as they often rapidly deteriorate. Another disadvantage that they employ is significantly slow analysis time because of a limitation of low flow rate due to thier high backpressure. New chromatographic materials are needed in order to determine interested analytes with high efficiency and good performance. Monolithic columns are resistant to extreme pH's however they are not as efficient as conventional silica based materials. Efficient monoliths should sufficiently be able to separate and determine small molecules as conventional columns. In this project we proposed to develop novel monolithic stationary phases implementing new high sensitivity methods to perform small molecule analysis using micro-HPLC and CEC techniques. For this we also focused on optimisation of new materials particularly a modified HILIC monolithic stationary phase to overcome the disadvantages.

In the first instance, a validation of micro-HPLC and CE instruments was established to ensure the capability of the capillary columns for the experiments. DiNa nano flow pump of micro-HPLC was successfully tested and worked efficiently. Acid and base separation experiments were performed employing EOF driving force for CEC. The results of these investigations were confirmed and

showed promise for the use of these instruments in nano-scale analysis for our research.

In the next step, screening and optimisation of the protocol for materials were investigated. Core materials i.e., a methacrylate HILIC monolith, SPE-co-EDMA was chosen as the stationary phase for this preliminary phase. Capillary was carried out studying factor affecting the column performance on HILIC polymerisation. The effect of HILIC fabrication such as polymerisation method (thermal vs microwave), time of polymerisation reaction (12 vs 24 hours), and initiator (AIBN vs V-501) was investigated to study column performance. The HILIC property was also studied using micro-HPLC employing different mobile phases (vary 3 proportions of organic solvent, acetonitrile and buffer solution). The optimisation of HILIC polymerisation and chromatographic system for HILIC monolithic analysis for micro-HPLC was successfully carried out as follow.

Column dimension; 100 µm (i.d.) x 375 µm (o.d.) x 300 mm (effective length)

Monoliths and conditions; poly SPE-co-EDMA (43: 57, %wt/wt), with 66.7 %wt/wt MeOH, 1%wt/wt AIBN, 12 hours thermal polymerisation time

Chromatographic system; ACN: 5 mM ammonium formate pH 3.0 (90: 10), 214 nm, flow rate 1000 nL/min

The optimised protocol of HILIC monolithic column successfully worked with micro-HPLC but was not applicable with CEC then an electroosmotic flow (EOF) of materials for CEC was modified and transferred to investigate the column

performance for the second phase. AMPS was chosen as an EOF provider for monolithic polymerisation while SCX particulate material was another EOF generator for the packed commercial HILIC column. These EOF promotors were added into the HILIC stationary phase for enhancing the EOF driving force for CEC experiments.

The preparation of new reversed-phase monoliths for improved performance on CEC, i.e., PEDAS modified with sulphonic acid groups i.e., AMPS column was successfully prepared and the column efficiency was evaluated in term of the numbers of the theoretical plates (*N*) when comparing with the micro-HPLC. The HILIC materials i.e., SPE-co-EDMA was modified with sulphonic acid groups i.e., AMPS. These new materials were manufactured with the optimised protocols and evaluated using both micro-HPLC and CEC. The presence of a sulphonic acid moiety in the new monolithic materials provided an EOF driving force for both RP test compounds and HILIC test compounds separations. The conclusion for these two Chapters confirmed that adding AMPS into the monolith polymerisation successfully worked with CEC. In addition, the modified HILIC monolith with sulphonic acid groups (SPE-co-EDMA-co-2% AMPS) clearly generated the best performance on both micro-HPLC and CEC.

The efficiency of our best performance monolithic column was compared with a packed commercial HILIC material column adding another EOF provider particulates (Atlantis HILIC, 3 µm with 10% SCX, 3 µm particle). The new process of making a packed commercial HILIC particle column was successfully developed using UV initiation for a mid-frit fabrication. The key finding in this Chapter was that

the best modified monolithic HILIC with sulphonic acid groups column still provided the best performance on both micro-HPLC and CEC when compared with the developed packed HILIC column. This was to confirm the best column, poly (SPE-co-EDMA-co-2% AMPS) monolithic column was beneficial to analyse small molecules.

Novel applications of HILIC (SPE-co-EDMA) monolithic column and modified HILIC with sulphonic acid groups (poly SPE-co-EDMA-co 2% AMPS) column were investigated using both micro-HPLC and CEC with chlorpheniramine maleate used as a test compound. These new methods for chlorpheniramine maleate tablets analysis using novel modified HILIC monolithic column by micro-HPLC and CEC were successfully validated. The method validation exhibited accomplished results with good performance on linearity, accuracy, precision and reproducibility as well as high sensitivity, specificity and pH stability. The modified HILIC with sulphonic acid groups (poly SPE-co-EDMA-co 2% AMPS) monolithic column showed excellent results on both micro-HPLC and CEC techniques with commercial chlorpheniramine maleate tablets determinations, but performed better when analysed with CEC rather than micro-HPLC. Our successful chlorpheniramine maleate determination methods by HILIC and modified HILIC monolithic columns are also original and pioneer employing HILIC mechanism to analyse chlorpheniramine by micro-HPLC and CEC. Using our developed method, both chlorpheniramine determinations in commercial ALLERCalm and Piriton tablets were successfully quantified within 5 minutes and passed the BP specification using our new methods on novel modified HILIC monolithic columns.

In summary, the achievement for our novel monolithic column is as following;

7.1.1 Sensitivity

From the method validation in Chapter 6, our new column was developed at nano-scale to enhance sensitivity of detection chlorpheniramine in picogram per injection unit. This was confirmed that our column has successfully achieved this.

7.1.2 Efficiency

It was proved from the experiments in many chapters that our new best modified HILIC with sulphonic acid moieties (poly SPE-co-EDMA-co-2% AMPS) monolithic column undoubtedly showed the significantly high the numbers of theoretical plates *(N)* especially using CEC method.

7.1.3 Environmental aspect

The success of CPM analysis using modified HILIC monolithic column by both micro-HPLC and CEC consumed only small amount of organic solvent. This showed that our methods used the green chemistry and they are environmental friendly.

7.1.4 pH stability

The analysis of chlorpheniramine in our research by CEC used an optimal condition at pH 11.0 evidently proving the extreme pH application for our modified HILIC monolithic column which is the advantage over the silica based materials.

This was distinctly that our column can be applied to a wide range of mobile phase for both micro-HPLC and CEC analysis.

7.1.5 Specificity

Markedly, the identification of chlorpheniramine in the commercial chlorpheniramine maleate tablets analysis in both micro-HPLC and CEC techniques showed the specificity of our column to quantify the drug.

7.2 Future works

Many interesting aspects referring from this research could be investigated further. Certainly the first follow-up is the application of the new modified HILIC column for biological sample analysis. Since good analysis reproducibility and efficiency was attained with our best monolithic column it would be considered acceptable to analyse bio-samples and will be tested for fingerprinting/metabolomics investigations with human urine samples. The second interesting aspect is a focusing effect from the research. Smith, et. al. [145] successfully published the focusing effect which enhanced incredibly high of the numbers of the theoretical plates (*N*) for their columns when evaluating column performance.

7.2.1 Modified HILIC monolithic column on application to human urine fingerprinting

The proof of concept for bio-sample analysis was investigated using a classical HPLC and CEC employing HILIC mechanism. The modified HILIC with EOF enhancer, poly SPE-co-EDMA-co-AMPS column was tested by injection of the 1/10 diluted human urine with the mobile phase; ACN: 5 mM ammonium acetate pH 5.8 (97.5: 2.5), background electrolyte; ACN: 5 mM ammonium acetate pH 5.8 (97.5: 2.5), normal polarity; + 30 kV, injection; 50 mBar X 1000 seconds, detection; 214 nm for CEC compared with a classical HPLC column (Luna-HILIC, 3 µm, 4.6 mm X 150 mm), mobile phase; ACN: 100 mM ammonium acetate pH 5.8 (97.5: 2.5), flow rate; 1 mL/min, injection volume; 20 µL, detection; 214 nm. The first try chromatograms are shown in Figure 7.1.

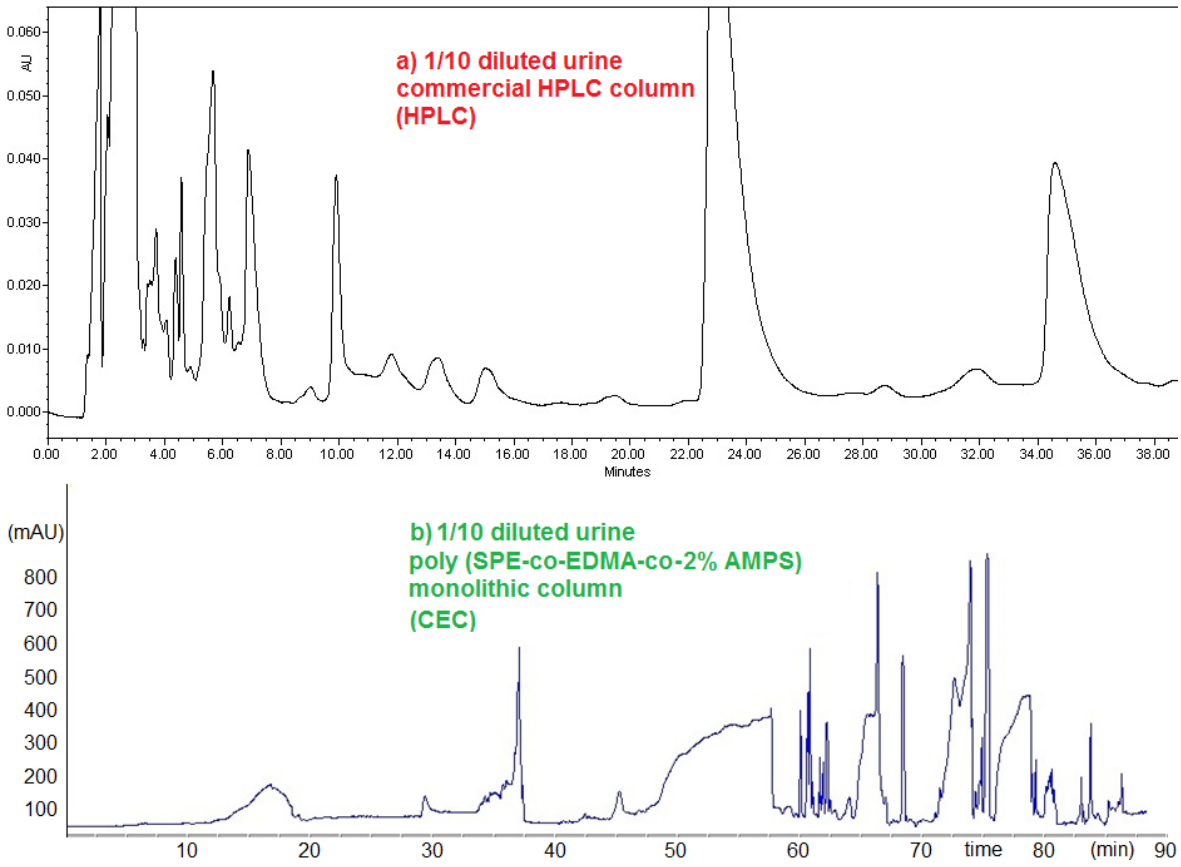


Figure 7.1 A comparison of chromatograms from 1/10 diluted human urine sample between a) a commercial Luna HILIC column by a classical HPLC and b) a poly (SPE-co-EDMA-co-2% AMPS) monolithic column using CEC

From these chromatograms, it is obviously seen that many polar metabolites from human urine sample were separated using both methods. For a classical HPLC in Figure 7.1 a), some polar metabolites were eluted from the HILIC column employing HILIC mechanism but still sensitivity and performance of the column are needed to be enhanced further. Using a mobile phase as a background electrolyte as ACN: 5 mM ammonium acetate pH 5.8 (97.5: 2.5) for our best modified HILIC (poly SPE-co-EDMA-co-2% AMPS) monolithic column exploiting HILIC mechanism with EOF providing moiety for CEC, many more human urine

metabolites can be separated with higher sensitivity and better in column performance. These interesting results show the possibility to extend the experiment for further investigation to acquire metabolic signatures in high throughput human urine studies using micro-HPLC and CEC techniques.

7.2.2 Focusing effect on the modified HILIC monolithic column

In 1995 Smith et. al. successfully published a focusing effect for their SCX columns. This gave their column performance million of the numbers of the theoretical plates (*N*) for tested analytes when evaluating their columns [145]. The proposed mechanism explanation on this phenomenon has been still debated for decades. For our attempts, it is noticeable that there were several times of this effect occurred on the HILIC test compounds separation especially a neutral small molecule, acrylamide when using a 100% ACN as a solvent for making the HILIC test mixture. The numbers of the theoretical plates (*N*) of acrylamide were significantly high when comparing to the toluene and thiourea under the same chromatographic conditions (ACN: 5 mM ammonium formate pH 3.0 (90: 10), 50 mBar X 1000 seconds, + 30 kV normal polarity, 214 nm) as shown in Table 7.1 and chromatograms in Figure 7.2. As it occurred only with a neutral acrylamide, this result requires further investigation for better understanding in mechanism to control the focusing effect.

Table 7.1 A solvent effect (focusing) on the numbers of the theoretical plates (*N*) of a modified HILIC with sulphonic acid groups, poly (SPE-co-EDMA-co-2% AMPS) monolithic column using CEC

Solvent for making a test mixture	Toluene	Acrylamide	Thiourea
	N (per metre) / [t_R (min)]	*N* (per metre) / [t_R (min)]	*N* (per metre) / [t_R (min)]
a) ACN: buffer pH 3.0 (90:10)	40754.6 ± 1194.4 [5.15 ± 0.04]	52097.5 ± 1663.7 [5.95 ± 0.05]	63116.9 ± 1894.7 [12.15 ± 0.09]
b) 100% ACN	41011.8 ± 1104.5 [5.11 ± 0.04]	254868.6 ± 3376.8 [5.90 ± 0.06]	63186.9 ± 1820.2 [12.06 ± 0.08]

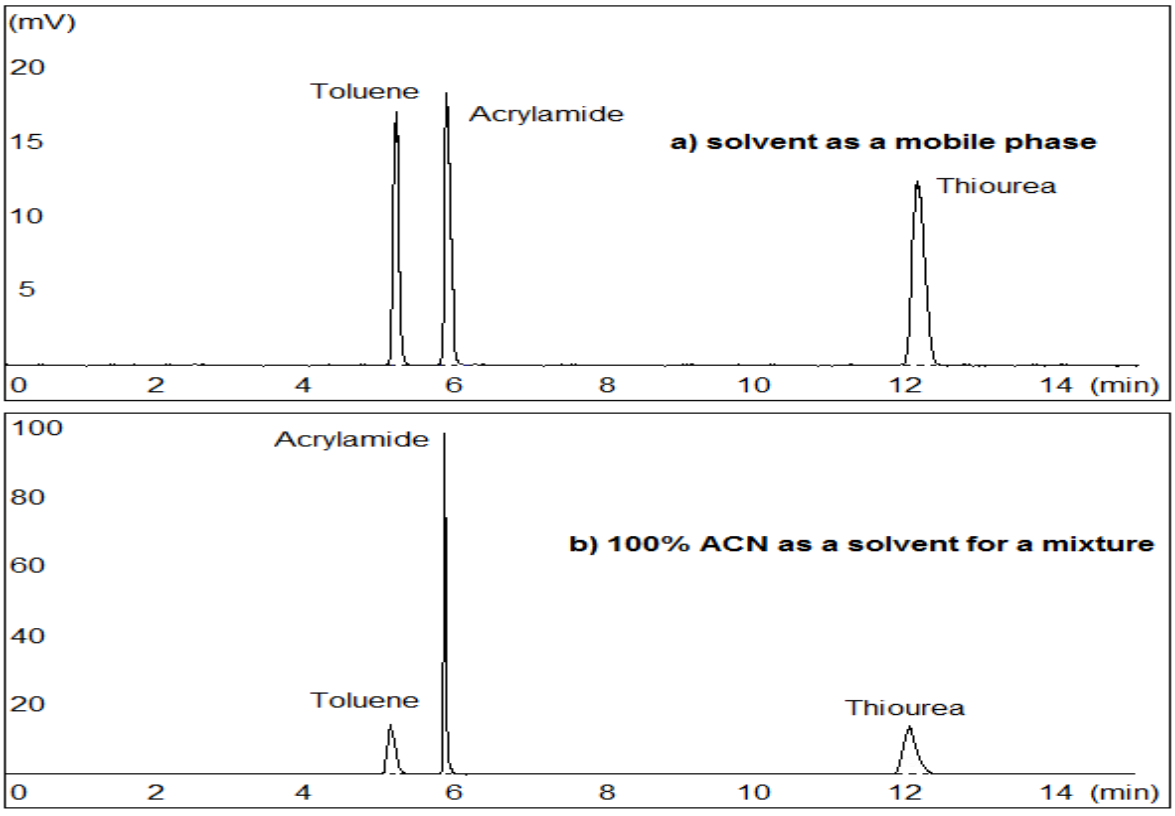


Figure 7.2 A comparison of CEC chromatograms on solvent effect (focusing) for a modified HILIC with sulphonic acid groups (poly SPE-co-EDMA-co-2% AMPS) monolithic column using a) solvent as a mobile phase and b) 100% ACN as a solvent for a mixture

More interesting evidence on focusing effect occurred with the separation of nitrogenous base nucleosides for example, adenine, cytosine and thymine including a polar (thiourea) and a non-polar (toluene) on our modified HILIC monolithic column using CEC. The potential results on this focusing effect occurred when using a normal polarity mode CEC with optimal CEC conditions (ACN: 5 mM ammonium formate pH 5.8 (97.5: 2.5), + 30 kV, 50 mBar X 1000 seconds, 214 nm). Millions of the numbers of the theoretical plates (*N*) of all nucleoside test compounds were carried out which were dramatically 80 times higher than those from a classical HILIC column (Luna-HILIC, 3 µm, 4.6 mm X 150 mm) with optimised chromatographic condition (ACN: 100 mM ammonium formate pH 5.8 (97.5: 2.5) mobile phase, 1.0 mL/min flow rate, 20 µl injection volume, and 214 nm UV detection) as shown in the Figure 7.3. This interesting investigation would be further studied and tested for the reproducibility.

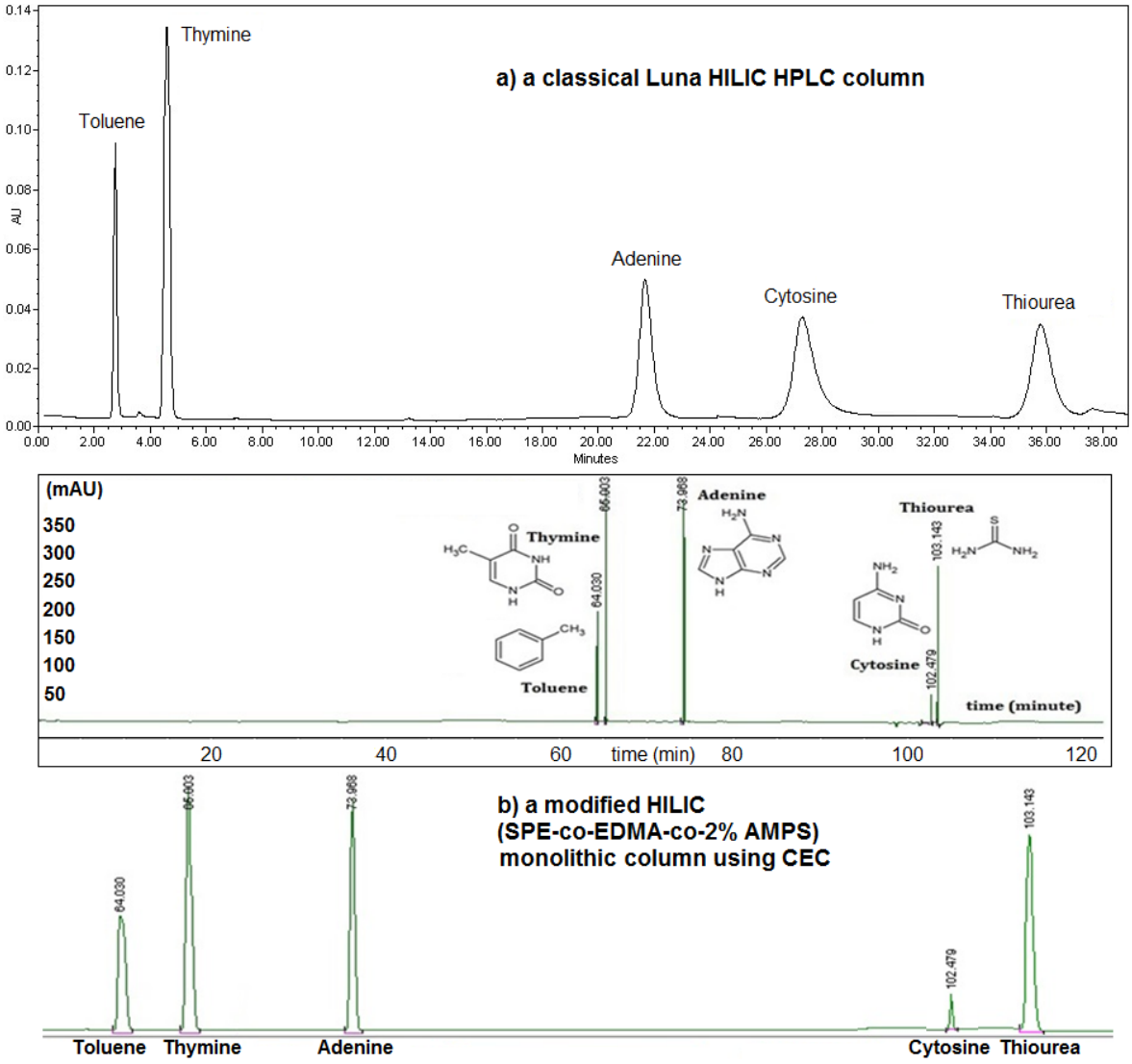


Figure 7.3 A comparison of nucleoside separation chromatograms on solvent effect (focusing) from a) a classical Luna HILIC HPLC column and b) a modified HILIC with sulphonic acid groups (poly SPE-co-EDMA-co-2% AMPS) monolithic column

Appendix

Presentations

1. A poster presentation at The 30th International Symposium on Chromatography; September 14th-18th, 2014, Salzburg, Austria; A John Dolphin Fellowship awarded (2014) by the Chromatographic Society (ChromSoc)

Topic; Mixed Monolithic-Packed Capillary Columns for Fingerprinting Polar Metabolites by micro-HPLC and CEC Studies.

2. A poster presentation at the 3rd Health Challenge Thailand Academic Conference, Thai Embassy, London, UK; April, 30th, 2013

Topic; Monolithic Column Manufacture and Column Performance Evaluation using micro-HPLC and Capillary Electrochromatography.

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